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A novel single-base deletion mutation of the *RUNX2* gene in a Chinese family with cleidocranial dysplasia

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ABSTRACT. We identified a disease-causing mutation of the *RUNX2* gene in a four-generation Chinese family affected with cleidocranial dysplasia (CCD). For mutation analysis, the coding region of *RUNX2* was sequenced with DNA from two patients and three unaffected family members. The *RUNX2* mutation was investigated in 50 normal controls by denaturing high pressure liquid chromatography. A heterozygous single-base deletion (c.549delC) of *RUNX2*, which predicts a termination site at the 185th codon and leads to a stop in the runt domain of RUNX2 protein, was detected in both patients but not in the three unaffected members of the family. This mutation was also not found in 50 controls and has not been reported previously. We demonstrated that a novel mutation (c.549delC) of *RUNX2* is associated with CCD in a Chinese family, adding to the repertoire of *RUNX2* mutations related to CCD.

Key words: CCD; *RUNX2*; c.549delC; Mutation analysis; DHPLC

INTRODUCTION

Cleidocranial dysplasia (CCD; MIM 119600), also known as cleidocranial dysostosis, is an autosomal dominant skeletal disorder characterized by wide cranial sutures with bulging calvaria, persistently open or delayed closure of the fontanelles, dental anomalies, hypoplasia or aplasia of the clavicles, short stature, cone-shaped thorax, wide pubic symphysis, short middle phalanx of the fifth fingers, and often vertebral malformation. It has been demonstrated that mutation of the runt-related transcription factor 2 gene (*RUNX2*; MIM 600211) on chromosome 6p21 is associated with CCD (Gelb et al., 1995; Mundlos et al., 1997). *RUNX2* encodes a transcription factor RUNX2, which is a major regulator of osteoblastic differentiation and bone formation. RUNX2 has been shown to bind to the DNA sequence element called RUNX-binding site (PyGPyGGT) and to regulate a number of bone-related genes (Li and Xiao, 2007). So far, over 96 mutations of *RUNX2* have been associated with CCD (Shen et al., 2009; Li et al., 2009; Zhang et al., 2010; Wang et al., 2010). Here, we report on a novel heterozygous mutation of *RUNX2,* associated with CCD in a Chinese family.

SUBJECTS AND METHODS

This study was approved by the university ethics committees, and informed consent was obtained from the patients and family members. Genomic DNA was extracted from peripheral blood leukocytes of 2 affected (III:7, IV:5) and 2 unaffected (III:3, III:5) individuals of a three-generation Chinese family with CCD (Figure 1) by means of standard protocols. All coding regions and the intron/exon boundaries were amplified by polymerase chain reaction (PCR). The primers were according to previous research (Cooper et al., 2001). A 20-µL reaction mixture included 30 ng genomic DNA and 10 µL Premix Ex Taq™ Hot Start Version (Takara). Amplifications were performed in a Perkin-Elmer thermal cycler (PTC-200). A 4-min initial denaturation at 94°C was followed by 30 cycles consisting of 30 s at 94°C, 30 s at 55° to 67°C and 40 s at 72°C, and a final elongation at 72°C for 10 min, ending with a holding period at 4°C. After checking by polyacrylamide gel electrophoresis, the PCR products were directly sequenced by the ABI Big Dye terminator cycle sequencing kit (ABI Biosystems) according to manufacturer instructions and run on an ABI 3100 sequencer. Sequencing results were analyzed using the DNASTAR® software program package.

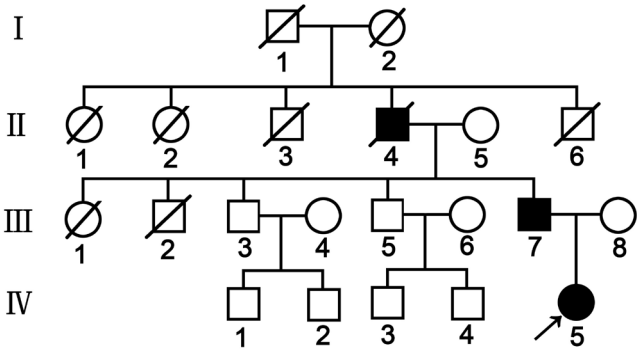

Figure 1. Pedigree of the Chinese family with CCD.

In order to exclude the possibility that the detected gene variation could be a polymorphism, DNA samples from 100 healthy control people were investigated by denaturing high performance liquid chromatography (DHPLC). For heteroduplex analysis, PCR products of the proband and 100 healthy controls were mixed 2:1 with that of a wild-type reference, respectively. The mixtures were denatured at 95°C for 6 min, and gradually reannealed from 95° to 25°C for a period of 60 min. The prepared samples (5 µL) were directly loaded into the autosampler of the automated DHPLC system, the WAVE® Nucleic Acid Fragment Analysis System, equipped with a DNASep cartridge (Transgenomic). The samples were run under partially denaturing conditions at 57.3°C, which was predicted by the navigator software. In the DHPLC system, fragments were screened for variation in retention time and chromatogram shape.

RESULTS

Clinical findings

The proband (IV:5) was an 18-year-old girl. Her facial appearance was typical of CCD, i.e., flattened face, broad forehead, frontal bossing, hypertelorism, low nasal bridge, and small maxilla (Figure 2a). She had dental anomalies including irregular forms of dentition, multiple supernumerary teeth in the maxilla and mandible, malocclusion, retention of primary teeth, and eruption failure of permanent teeth (Figure 2b-c). The skull X-ray films showed patent fontanelles, wide cranial sutures, and protruding mandible (Figure 2d-e). Her hypoplastic clavicles resulted in narrow and sloping shoulders, as well as cone-shaped thorax (Figure 2f). According to the phenotypic characteristics described above, the proband was diagnosed with CCD in the Department of Stomatology of Xiangya Hospital, Central South University, China. The proband's father (III:7) also displayed features of CCD, including patent fontanelles, wide cranial sutures, broad forehead, frontal bossing, flattened face, small maxilla, protruding mandible, dental anomalies and hypoplastic clavicles. The proband's mother did not show phenotypic signs of CCD.

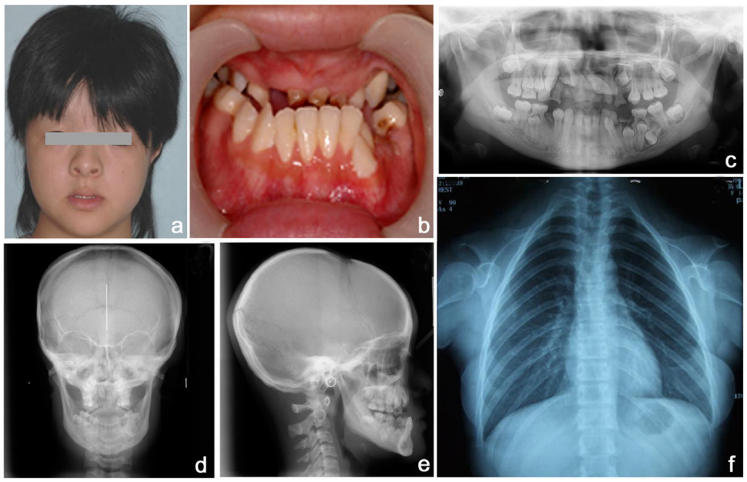

Figure 2. Photographs showing the CCD phenotypic characteristics of the proband.

Genetic characterization

Direct sequencing of the PCR products of the proband showed a heterozygous C deletion at nucleotide 549 of *RUNX2* (Figure 3A), which is a frameshift mutation and leads to a termination site at the 185th codon (p.Asn183fsX185) (Figure 3B). The predicted protein product lacks 337 amino acids, including the NLS and the PST domain (Figure 3C). The proband's father (III:7) is heterozygous for this mutation, which is not found in 2 unaffected family members (III:3, III:5), indicating that the mutation cosegregates with CCD in the family.

To further confirm that the mutation c.549delC in *RUNX2* is responsible for CCD, we investigated this mutation in 100 healthy controls by DHPLC. The chromatograms of the proband and her father showed a heteroduplex, while the wild-type reference and 100 controls showed a homoduplex (Figure 3D), indicating that this mutation does not exist in 100 unrelated healthy controls.

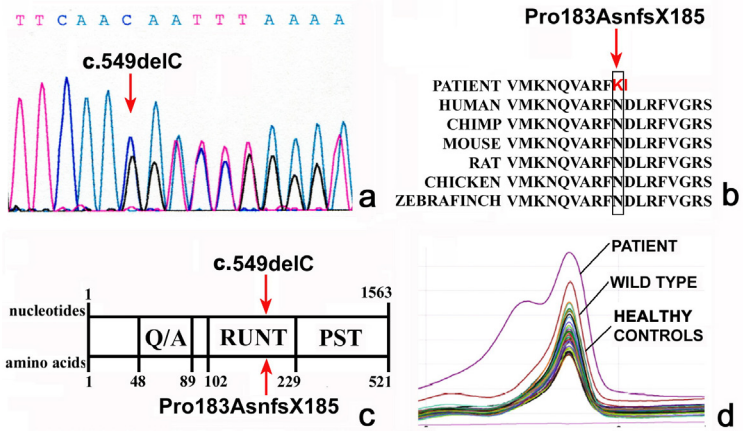

Figure 3. Genetic characterization of the CCD family. (**a**) Sequencing result of the proband showing a single-base deletion (c.549delC) in *RUNX2*. (**b**) Evolutionary conservation of amino acids: the box showing loss of the conserved asparagine residue and truncation of protein in the patient. (**c**) Relative position of the mutation in *RUNX2*. (**d**) DHPLC showing heteroduplex in the patient but homoduplex in the wild-type reference and healthy controls.

DISCUSSION

Skeletal growth of CCD patients is affected in many ways. Bones derived from intramembranous ossification, such as the cranium and the clavicles, are most prominently affected. The prominent abnormalities of the skull in CCD include persistently wide-open or delayed closure of fontanelles, which may remain open throughout life, and a wide-open metopic suture with bulging calvaria, which results in the separation of the frontal bones by a metopic groove. The typical facial appearances are flattened face, broad forehead, frontal bossing, hypertelorism, low nasal bridge, small maxilla, and protruding mandible. Up to 94% of patients with CCD have abnormal dentition, including multiple supernumerary teeth, retention of primary teeth, eruption failure of permanent teeth, malocclusion, irregular forms of dentition, wide spacing in the lower incisor area, supernumerary tooth germs, parallel-sided ascending rami,

and cysts in their gums that usually form around extra teeth (McNamara et al., 1999; Cooper et al., 2001; Golan et al., 2003, 2004). Most patients have abnormal clavicles ranging from complete absence to hypoplastic or discontinuous clavicles, and hypoplastic clavicles commonly result in narrow, sloping shoulders that can be apposed at the midline. Other skeletal abnormalities include cone-shaped thorax, wide pubic symphysis, short middle phalanges, scoliosis, osteoporosis, genua valga and pes planus (Cooper et al., 2001). Although there is a wide spectrum of phenotypic variability, ranging from primary dental anomalies to all CCD features plus osteoporosis, no clear phenotype-genotype correlation has been established (Otto et al., 2002). From a study using a new mouse model with a hypomorphic Runx2 mutant allele (Runx2^{neo7}), Lou et al. (2009) suggested that there is a critical gene dosage requirement of functional Runx2 for the formation of intramembranous bone and that the range of bone phenotypes in CCD patients is attributable to quantitative reduction in the functional activity of RUNX2.

Over 96 mutations of *RUNX2* have been reported to be associated with CCD so far, including missense, deletion, insertion, nonsense, and splice-site mutations, as well as chromosomal translocations (Shen et al., 2009; Li et al., 2009; Zhang et al., 2010; Wang et al., 2010). In the present study, a single-base deletion (c.549delC) in *RUNX2* was identified in a Chinese CCD family. The 1-bp deletion affects the third base pair of the 183rd codon (out of 522), creating a frame shift that predicts a termination site at the 185th codon (p.Asn183fsX185) and leads to the truncation of the last 337 amino acids (65% of the polypeptide). The mutation cosegregated with the disease phenotype in the CCD family and was not found in 100 unrelated healthy controls, indicating that the c.549delC of *RUNX2* is the causative mutation of CCD in this family.

The runt-related transcription factor 2 (RUNX2) encoded by *RUNX2* is a major transcription factor involving osteoblast differentiation and bone formation. The mouse models homozygous for the mutated *RUNX2* allele showed complete lack of ossification of the skeleton and died after birth without breathing (Komori et al., 1997), and the mice carrying one mutated *RUNX2* allele showed specific skeletal abnormalities characteristic of CCD (Otto et al., 1997). *RUNX2* is one of the three human "runt domains" containing genes and RUNX2 contains an N-terminal stretch of consecutive polyglutamine and polyalanine repeats (Q/A domain), a runt domain and a C-terminal proline/serine/threonine-rich (PST) activation domain (Figure 3C). The majority of *RUNX2* causative mutations in patients with classic CCD affect the runt domain of the protein. The runt domain is a 128-amino-acid polypeptide that has the ability to mediate DNA binding and protein heterodimerization with the CCAAT/enhancer-binding protein (C/EBP). The RUNX2 and C/EBP act synergistically to activate the bone-specific osteocalcin genes, such as *osteocalcin*, expression (Gutierrez et al., 2002). The C-terminal PST domain is thought to be the transcription activation domain, which is involved in functional interactions with various other transcription factors, co-activators and co-repressors (Thirunavukkarasu et al., 1998). In addition, a nuclear-localization signal (NLS) was located at the junction of the runt and PST domains (Figure 3c). The NLS is a short basic stretch of nine amino acids [amino acids 221-229 in RUNX2 (VDGPREPRR)] that is necessary for nuclear localization of the protein (Thirunavukkarasu et al., 1998). Analysis of the subcellular distribution of RUNX2–GFP fusion proteins after transient transfection into 293A cells indicated that the two frameshift mutations-214fs and 172fs-completely abolished the function of NLS, rendering the protein unable to quantitatively accumulate in the nucleus (Zhang et al., 2010). Likewise, the single-base deletion mutation (c.549delC) identified in the present study occurs in the highly conserved runt domain and leads to a termination site at the

185th codon (p.Asn183fsX185), which is expected to cause the deletion of NLS and the PST domain and to result in a protein unable to accumulate in the nucleus, producing the classic CCD phenotype in our patients due to haploinsufficiency.

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