Mutations in the RUNX2 Gene in Chinese Patients with Cleidocranial Dysplasia

Dongying Xuan,1 Shi Li,2 Xiong Zhang,1 Fei Hu,1 Lixin Lin,1 Chunxian Wang,1 and Jincai Zhang1
1Department of Periodontology, Guangdong Provincial Stomatological Hospital, Southern Medical University, Guangzhou, China; 2Department of Endodontics, College of Stomatology, Fourth Military Medical University, Xi’an, Shaanxi, China

Abstract. Cleidocranial dysplasia (CCD) is an autosomal dominant inheritable skeletal disease caused by heterozygous mutations in an osteoblast-specific transcription factor, RUNX2. Mutational analyses of RUNX2 were done on 4 unrelated Chinese patients with CCD. One nonsense and 3 missense mutations were detected, including one novel mutation, a heterozygous G to C transition mutation at nucleotide 475 in exon 2, which converts glycine to arginine at codon 159 (G159R). Two mutations, R225W and R391X, were reported in Chinese patients with CCD for the first time. Our findings show that R225 mutations interfere with nuclear accumulation of RUNX2 protein, and that a lack of nuclear RUNX2 protein accumulation is at least one of the causes of haploinsufficiency in these cases. Body stature was significantly reduced in the 3 male and 1 female cases. The cases all had malformations of the tarsometatarsal joints. In 1 case, the humeroulnar joints and humeroradial joints were abnormal, and the elbow looked like a triangle. The data suggest that an impaired runt domain contributes to the short stature of CCD patients. We postulate that RUNX2 influences joint formation by affecting the differentiation pathways of chondrocytes and osteoblasts.

Keywords: cleidocranial dysplasia, RUNX2 mutation, joint malformations, RUNX2 protein localization

Introduction

Cleidocranial dysplasia (CCD; MIM #119600) is a skeletal disorder with autosomal-dominant inheritance. The clinical hallmarks of CCD include rudimentary or absent clavicles, delayed closure of cranial fontanels and sutures, Wormian bones, frontal bossing, supernumerary and late erupting teeth, and wide pubic symphysis [1]. The phenotypic spectrum ranges from mildly affected individuals with only dental abnormalities to severely affected cases with generalized osteoporosis; tooth anomalies and some degree of clavicular hypoplasia are the most consistent features of the disease [2].

The locus for CCD has been mapped to chromosome 6p21 where the responsible runt-related gene 2 (RUNX2) has been located [3,4]. RUNX2 is also referred to as polyomavirus enhancer binding protein 2 (PEBP2) and murine leukemia virus enhancer core-binding factor (CBF)[4,5], and it encodes 1 of the 3 distinct mammalian α-subunits of PEBP2/CBF. The α-subunit harbors the DNA binding ability in an evolutionally conserved 128-aa region termed the runt domain, which shares high homology with the products of the Dro sophila genes runt [6] and lozenge [7].

The CCD syndrome was described accurately for the first time by Scheuthauer in 1871 [8]. One of the most colorful families, descendants of a Chinese named Arnold, was described by Jackson in 1951 [9]. He traced 356 members of this family of whom 70 were affected with the “Arnold head.” Clinical and genealogical studies of the affected
descendants of the Chinese Arnold were continued in South Africa, but investigations of Chinese subjects with CCD have rarely been reported from China. The prevalence and range of \textit{RUNX2} mutations in Chinese cases with CCD are not well documented.

Here, we report 4 different types of heterozygous mutations in the \textit{RUNX2} gene in 4 unrelated CCD cases from China who display variable clinical manifestations, and we delineate the molecular basis of their dysfunction. The present results provide further genetic evidence that mutations of the \textit{RUNX2} gene are responsible for CCD and that heterozygous loss of function is sufficient to produce the characteristic clinical findings.

**Materials and Methods**

Four unrelated families with the clinical diagnosis of CCD (for diagnostic criteria, see reference [1]) were investigated, including the unaffected parents and siblings of the CCD patients. Informed consent was obtained from all individuals. Radiological examinations for osseous malformations included the entire body. Lateral cephalometric, antero-posterior, and panoramic radiographs were taken to examine the skeletal morphology of the skull and face and to evaluate dental development.

**Mutation analysis of \textit{RUNX2}**. Genomic DNA was extracted from whole blood samples by using the QLAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Exons 0-7 of the \textit{RUNX2} gene were amplified by PCR from genomic DNA using the combination of intron- and exon-specific primers reported by Quack et al [2] (Table 1). PCR amplification was done in a thermal cycler. The PCR conditions were optimized and the amplification products were checked by agarose gel electrophoresis and purified with the PCR purification kit (Agarose Gel DNA Purification Kit) according to the manufacturer’s instructions. To resolve ambiguities in the primary sequence, products of an independent PCR reaction were subcloned by use of the pMD20 TA vector (TaKaRa) and sequenced using an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were analysed using the BLASTN (BLAST nucleotide) program (http://www.ncbi.nlm.nih.gov/BLAST). Mutation numbering designated the ATG codon as number 1 [3].

**Subcellular localization of mutant \textit{RUNX2} protein**. To transiently express green fluorescent protein fusions, the runt domain, including the putative nuclear localization signal (NLS), was subcloned into pEGFP-C1 (Clontech) and the mutations R255Q and R255W were introduced into the resulting plasmids. All plasmids were fully sequenced to exclude any additional mutations. National Institutes of Health (NIH) 3T3 cells were transiently transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. At 48 hr after transfection, the 3T3 cells were fixed with 4% paraformaldehyde for 15 min and washed 3 times with PBS. Subcellular localization of the RUNX2-GFP fusion protein was determined by fluorescence microscopy.

**Results**

Four unrelated Chinese families with the clinical diagnosis of CCD were included in this study.
Fig. 1. Radiological findings and mutational analysis in case 1. (A) Chest radiograph showing small, bell-shaped thorax with short and oblique ribs, and bilateral clavicular hypoplasia with 2 separate fragments. (B) Foot radiograph showing abnormal tarsal bones and tarsometatarsal joints that slope laterally. (C) Lateral cephalometric radiograph showing maxillary hypoplasia and relatively hyperplastic mandible. (D) Occipitomental radiograph showing hypoplasia of the maxillary sinus and zygomatic bones. (E) Panoramic view showing retention of deciduous teeth and impacted permanent teeth. (F) Sequence analysis of the mutation. Sequencing of exon 2 of $RUNX2$ alleles shows the heterozygous mutant allele with G>C missense mutation in the runt domain (475 G>C) and the wild-type allele (WT; the sequence is from the patient’s normal father). The arrows indicate the features mentioned in the respective legends.
Fig. 2. Clinical and radiological findings and mutational analysis in case 2. (A) Chest radiograph that shows right clavicular hypoplasia with 2 separate fragments. (B) Panoramic view that shows the unerupted and supernumerary teeth. A dentigerous cyst is located at the left second premolar. (C) Intraoral image showing malocclusion and amelogenesis hypoplasia. (D) Occipitomental radiograph shows that the zygomatic bones were almost absent. (E) Lateral cephalometric radiograph that shows significant prognathism. (F) Sequence analysis of mutation. Sequencing of exon 3 of RUNX2 alleles shows the heterozygous mutant allele with G>A missense mutation in the runt domain (674 G>A) and the wild-type allele (WT; the sequence is from the patient’s normal father). The arrows indicate the features mentioned in the respective legends.
Fig. 3. Radiological findings and mutational analysis in case 3. (A) Chest radiograph shows the cone-shaped thorax and the absence of the bilateral acromial ends, which were represented by fibrous pseudarthrosis. (B) Panoramic view shows supernumerary teeth in the molar region and impacted permanent teeth with delayed development and abnormal roots. (C) Occipitomental radiograph shows that the zygomatic bones were almost absent. (D) Sequence analysis of mutation. Sequencing of exon 7 of RUNX2 alleles shows the heterozygous mutant allele with C>T nonsense mutation (1171 C>T) and the wild-type allele (WT; the sequence is from the patient’s normal father). (E) Right foot radiograph shows abnormal tarsal bones and tarsometatarsal joints that slope laterally. (F) Lateral cephalometric radiograph shows a counter clockwise rotation of the mandible that results in mandibular protrusion. The nasal bone was almost absent. The arrows indicate the features mentioned in the respective legends.
Fig. 4. Radiological findings and mutational analysis in case 4. (A) Chest radiograph shows a cone-shaped thorax, complete absence of the right clavicle, and hypoplasia of the left acromial end. (B) Radiograph of right elbow joint shows that the ulna and radius both leaned to the radialis so that the humeroulnar and humeroradial joint were abnormal. (C) Foot radiograph shows absence of the distal phalanx of the first toe. (D) Lateral cephalometric radiograph shows maxillary hypoplasia and significant mandibular protrusion. The nasal bone was almost absent. (E) Sequence analysis of mutation. Sequencing of exon 3 of RUNX2 alleles shows the heterozygous mutant allele with C>T missense mutation in the runt domain (673 C>T) and the wild-type allele (WT; the sequence is from the patient’s normal father). (F) Panoramic view shows retention of the deciduous dentition and supernumerary teeth located in premolar region. The arrows indicate the features mentioned in the respective legends.
Table 2. Summary of CCD mutants in this study.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (yr)</th>
<th>Gender (M/F)a</th>
<th>Delayed closure of suturesb</th>
<th>Hypoplastic claviclesc</th>
<th>Height SD scores of affected subjects</th>
<th>Mutations</th>
<th>Nucleotide</th>
<th>Codon</th>
<th>Type</th>
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<tr>
<td>1</td>
<td>29</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>-4.01</td>
<td>475G → C</td>
<td>G159R</td>
<td>Missense</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>-3.42</td>
<td>674G → A</td>
<td>R225Q</td>
<td>Missense</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>M</td>
<td>+</td>
<td>++</td>
<td>-1.21</td>
<td>1171C → T</td>
<td>R391X</td>
<td>Nonsense</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>M</td>
<td>+</td>
<td>++</td>
<td>-1.78</td>
<td>673C → T</td>
<td>R225W</td>
<td>Missense</td>
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</tr>
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</table>

a F = female; M = male  
b + = present  
c + = hypoplastic clavicle(s); ++ = complete absence of the clavicle(s)

Fig. 5. Subcellular localization of mutant RUNX2 protein. Fluorescence photomicrographs of RUNX2-GFP fusion proteins expressed in NIH-3T3 cells show the different subcellular localization of wild-type RUNX2 (A), mutant R225Q RUNX2 (B), and mutant R225W RUNX2 (C). Panel (D) shows the corresponding brightfield photomicrograph of (B).
There was no parental consanguinity. A CCD phenotype was defined by the triad of hypoplastic clavicles and delayed closure of the anterior fontanelle in addition to classic craniofacial features. Only case 2 was familial, and his mother and a younger brother were affected by CCD. The skeletal anomalies and oral manifestations of the syndrome were variable in the affected families, which is consistent with the literature [10,11].

The four proband cases display variable manifestations besides the characteristic triad (Figs. 1-4). According to the cephalometric analyses, cases 2 and 3 showed increase of the superior facial depth (N-S) and middle facial depth (A'-Ptm'), but their maxillae were relatively normal (SNA, S'-Ptm'). Their mandibular dimensions were far greater than those in the normal group (Pog-Go', SNB, Go-Co), and the posterior and anterior facial heights were both greater (S-Go/N-Me). These changes resulted in mandibular prognathism. Case 4 showed reduction of the superior facial depth (N-S) and middle facial depth (A'-Ptm'), and protrusion of the maxilla (SNA, S'-Ptm'). In all 4 cases, these changes caused counter clock-wise rotation of the mandible, resulting in various degrees of mandibular protrusion.

**Characterization of heterozygous mutations.** To identify mutations in the \(\text{RUNX2}\) gene in the CCD cases, we analyzed genomic DNA. In the 4 families examined, we detected 4 heterozygous mutations (Table 2). A novel missense mutation was included within the exon 2 in case 1, which like many CCD mutations occurs in the runt domain [12]. This subject’s healthy parents did not carry the same mutation. This mutation converted glycine to arginine at codon 159 (G159R) (Fig. 1F). Detailed analyses of this mutation will be reported elsewhere (manuscript in preparation). Although the other 3 mutations have previously been reported [12-14], the R225W and R391X mutations were detected in Chinese cases with CCD for the first time.

**Subcellular localization of mutant RUNX2 protein.** To study the effect of mutations on nuclear localization of \(\text{RUNX2}\) protein, fusion proteins were constructed between green fluorescent protein and \(\text{RUNX2}\). The constructs were transiently transfected into mouse fibroblast NIH-3T3 cells. Wild-type \(\text{RUNX2}\) protein was detected exclusively in the nucleus (Fig. 5A). However, the R225Q and R225W mutants showed dual localization in both the cytoplasm and the nucleus (Fig. 5B,5C).

**Discussion**

**Clinical manifestations.** With more cases of CCD reported, early diagnosis of CCD is not difficult. In a patient with hypoplastic clavicles, open fontanelles, and supernumerary teeth, the diagnosis of CCD is evident and few other conditions need to be considered. However, the clinical manifestations of CCD are variable, even within families, and the phenotype may be incomplete, lacking one or two signs of the characteristic triad. There are other syndromes with congenital clavicular hypoplasia or agenesis, such as congenital pseudarthrosis of the clavicle (MIM 118980), pyknodysostosis (MIM 265800), and Yunis-Varon syndrome (MIM 216340), which may resemble CCD [1]. However, distinguishing these syndromes from CCD is not difficult if attention is paid to the basic clinical characteristics. For example, the presence of osteosclerosis and the absence of supernumerary teeth are sufficient to distinguish CCD from pyknodysostosis.

Supernumerary teeth and impacted permanent teeth are among the common features of CCD [15-17]. In case 1, impacted permanent teeth were situated in the canine and premolar region. In cases 2 and 4, the extra teeth were located mainly in the anterior and premolar region, as frequently occurs [18,19]. Case 3 had extra teeth in the molar region (at least four molars), which has rarely been reported. Moreover, the impacted molars and extra molars had abnormal roots with delayed development, and the erupted first molars had relatively normal roots. It seems that abnormal root development may be one cause of eruption failure of permanent teeth in CCD cases.

\(\text{RUNX2}\) is required for osteoblast differentiation and chondrocyte maturation during endochondral bone formation [1]. Haploinsufficiency of \(\text{RUNX2}\) is linked to decreased chondrocyte hypertrophy and to the altered expression and distribution of type X collagen in CCD [20]. All of the cases in...
the present study showed malformation of tarso-metatarsal joints to a certain extent. In case 4, the ulna and radius both leaned to the radialis so that the humeroulnar joints and humeroradial joints were abnormal and the elbow looked like a triangle. Thus we infer that RUNX2 plays a role in joint formation by affecting chondrocyte and osteoblast differentiation.

Genotype-phenotype relationships. In a study of genotype-phenotype correlations in CCD cases, Yoshida et al [21] reported a substantial difference of height SD scores between two groups of patients with or without mutational impairment of the runt domain. The average stature was much lower (SD -2.56) in the impaired group of 27 cases versus the intact group of 4 cases (SD -0.55). Correlation was also noted between the height SD scores and the number of supernumerary teeth. In our study, the runt domain was mutationally impaired and body stature was markedly reduced in all 4 cases, which supports the view that an impaired runt domain may contribute to the short stature of CCD cases.

Residue R225 is important for the function of RUNX2 protein. Amino acid R225 is located in a stretch of basic amino acids at the carboxy terminus of the runt domain of RUNX2 protein. Mutations that affect R225 have been frequently identified in CCD patients [2,12,14,22]. In the present study, the R225Q and R225W mutants showed RUNX2 protein localization in both the cytoplasm and the nucleus. These results are consistent with evidence that this area contains a nuclear localization signal (NLS) and that mutations affecting R225 impair nuclear uptake of RUNX2 protein [2]. Lack of nuclear RUNX2 accumulation is at least one of the causes of haploinsufficiency in CCD cases.

Deletion analyses have suggested that the RUNX2 gene contains additional elements that promote nuclear localization, as coarsely mapped to the N-proximal half of the runt domain (positions 93-158) and the C-terminal region that spans amino acids 272-502, in addition to the NLS on the C-terminal border of the runt domain [23,24]. A mutational change in one or more of these elements would reduce nuclear localization to some extent, but total inhibition would be unlikely unless all of the elements are physically destroyed or structurally perturbed.

In the present study, mutations of the RUNX2 gene were detected in 4 Chinese CCD cases. This study documents the variable clinical features of CCD cases, including characteristics that have rarely been reported. The RUNX2 gene may be involved in several steps during tooth development, but why mutations in RUNX2 cause supernumerary teeth is not obvious, and the loss of control of tooth number in CCD merits further study.

Acknowledgements

We thank the patients and their families whose cooperation made this study possible, as well as Drs. XianFeng Wan, Ji Kuang, and Zhi Yong Zhang for assistance in radiographic analyses.

References


