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RUNX2 mutations in cleidocranial dysplasia

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ABSTRACT. The runt-related transcription factor 2 gene (*RUNX2*), which is also known as *CBFA1*, is a master regulatory gene in bone formation. Mutations in *RUNX2* have been identified in cleidocranial dysplasia (CCD) patients. CCD is a rare autosomal dominant skeletal dysplasia that is characterized by delayed closure of cranial sutures, aplastic or hypoplastic clavicle formation, short stature, and dental anomalies, including malocclusion, supernumerary teeth, and delayed eruption of permanent teeth. In this study, we recruited three *de novo* CCD families and performed mutational analysis of the *RUNX2* gene as a candidate gene approach. The mutational study revealed three disease-causing mutations: a missense mutation (c.674G>A, p.Arg225Gln), a frameshift mutation (c.1119delC, p.Arg374Glyfs*), and a nonsense mutation (c.1171C>T, p.Arg391*). Clinical examination revealed a unique dental phenotype (no typical supernumerary teeth, but duplication of anterior teeth) in one patient. We believe that this finding will broaden the understanding of the mechanism of supernumerary teeth formation and CCD-related phenotypes.

Key words: *RUNX2*; Cleidocranial dysplasia; Supernumerary teeth; Mutation

INTRODUCTION

Cleidocranial dysplasia (CCD; OMIM 119600) is a rare autosomal dominant disease, which is characterized by delayed closure of cranial sutures, aplastic or hypoplastic clavicle formation, short stature, and dental anomalies, including malocclusion, supernumerary teeth, and delayed eruption of permanent teeth (Mundlos, 1999). The penetrance is complete, but the expressivity is variable in terms of the stature and number of supernumerary teeth (Otto et al., 2002; Ryoo et al., 2010).

Heterozygous mutations in the runt-related transcription factor 2 gene (*RUNX2*), the master regulator of bone formation, causes CCD (Lee et al., 1997; Mundlos et al., 1997). *RUNX2* is also known as core binding factor A1 (*CBFA1*) and it is located on chromosome 6p21 (Bae et al., 1993; Ducy et al., 1997). It is generally believed that the haploinsufficiency or loss of function of RUNX2 underlies the mechanism of CCD pathogenesis (Quack et al., 1999; Kim et al., 2006).

In this study, we recruited three *de novo* CCD families and screened for *RUNX2* as a candidate gene approach. Mutational analysis revealed three mutations in *RUNX2* and the clinical characteristics related to supernumerary teeth were analyzed.

MATERIAL AND METHODS

Identification and enrollment of human subjects

This study was independently reviewed and approved by the Institutional Review Board at the University of Istanbul and the Seoul National University Dental Hospital. Experiments were undertaken with the understanding and written consent of each subject according to the Declaration of Helsinki.

Polymerase chain reaction (PCR) and sequencing

Genomic DNA was isolated from peripheral whole blood using the QuickGene DNA whole blood kit S with QuickGene-Mini80 equipment (Fujifilm, Tokyo, Japan). DNA purity and concentration was quantitated by spectrophotometry, as measured by the OD_{260}/OD_{280} ratio. Mutational analysis was performed for the exons and exon-intron sequences of *RUNX2*, according to a previous report (Zhang et al., 2000a) using the HiPi DNA polymerase premix (ElpisBio, Taejeon, Korea). PCR products were purified using a PCR Purification Kit (ElpisBio). DNA sequencing was performed at the DNA sequencing center (Macrogen, Seoul, Korea). The reference sequence used for mutation numbering was the longest transcript (NM_001024630.3) of *RUNX2* with the second ATG start codon (starting MASNS).

RESULTS

Patient 1

Mutational analysis of *RUNX2* revealed a single nucleotide change (c.674G>A)

(Quack et al., 1999; Otto et al., 2002). This nucleotide change resulted in a missense mutation (p.Arg225Gln) in the runt domain of the RUNX2 protein (see Figure 1A). The proband was a 17-year-old boy with mixed dentition (see Figure 1B and C). Radiographic examination showed bilateral hypoplastic clavicles and maxillary hypoplasia (see Figure 1D and E). Deciduous teeth were retained and eruption failure of permanent dentition and supernumerary teeth were noted.

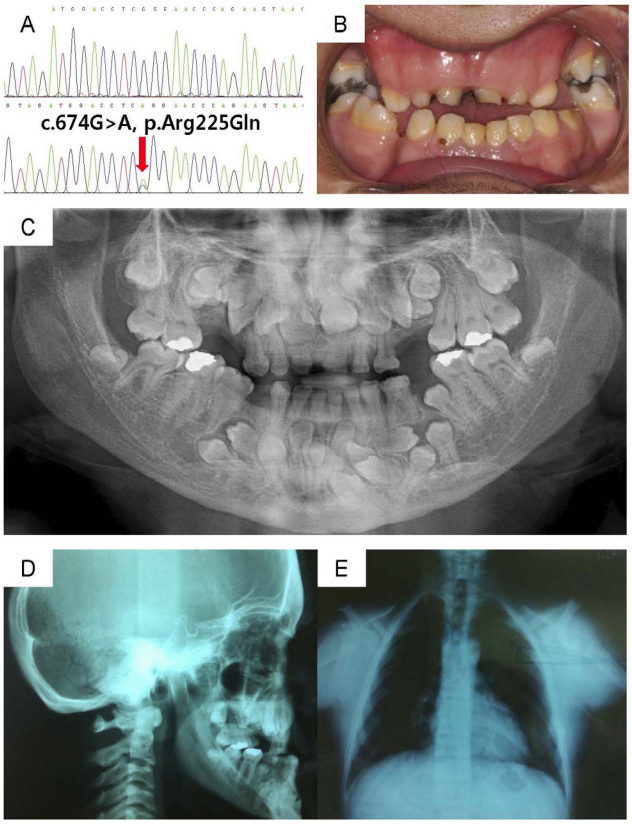

Figure 1. Mutational analysis, clinical photo, and radiologic examination of Patient 1. **A.** Mutational analysis reveals a single nucleotide change (c.674G>A, p.Arg225Gln). **B.** Frontal clinical photo. Anterior deciduous dentition is retained. **C.** Panoramic radiograph shows retention of eruption failure of permanent dentition and supernumerary teeth. **D.** Maxilla is hypoplastic. **E.** Both clavicles are hypoplastic.

Patient 2

Mutational analysis revealed a single nucleotide deletion mutation (c.1119delC) in the proband (Lin et al., 2011) (see Figure 2A). This mutation was located in the last exon, which indicates that it would escape nonsense-mediated mRNA decay. The predicted protein (p.Arg374Glyfs*) lacked the C-terminus domain of the normal RUNX2 protein. The proband was a 12-year-old boy with an anterior crossbite (see Figure 2B). Both clavicles were

severely hypoplastic and supernumerary teeth were impacted in the jawbones (see Figure 2C, D, and E).

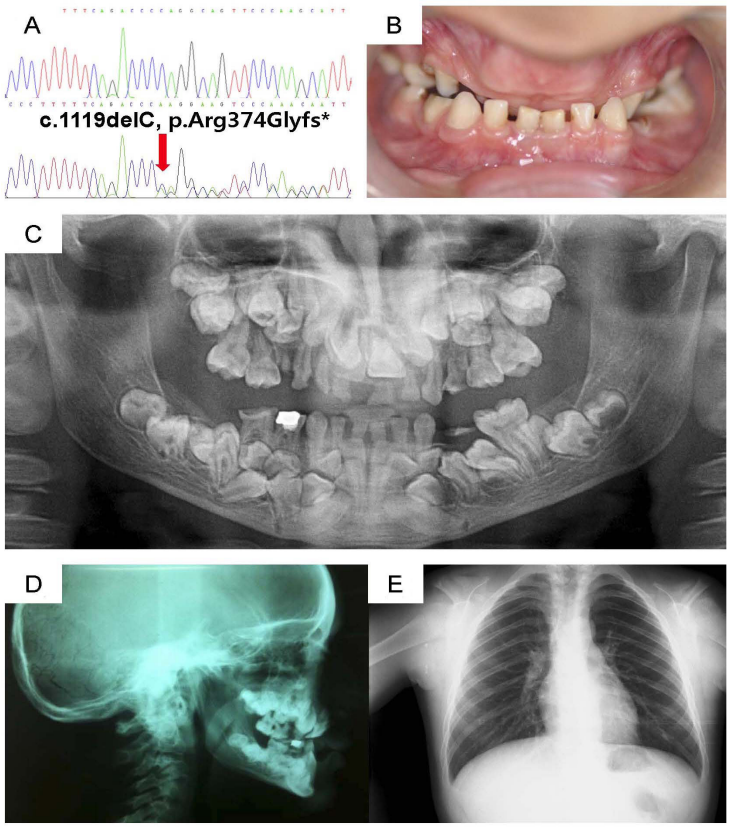

Figure 2. Mutational analysis, clinical photo, and radiologic examination of Patient 2. **A.** Mutational analysis reveals a single nucleotide deletion (c.1119delC, p.Arg374Glyfs*). **B.** Frontal clinical photo shows anterior crossbite. **C.** Panoramic radiograph shows retention of eruption failure of permanent dentition and supernumerary teeth. **D.** Paranasal sinuses are hypoplastic, but maxilla is not hypoplastic. **E.** Both clavicles are severely hypoplastic.

Patient 3

Mutational analysis revealed a single nucleotide change (c.1171C>T) in the proband (Zhang et al., 2000b) (see Figure 3A). Because this mutation was also located in the last exon, the predicted protein (p.Arg391*) lacked the C-terminus domain of the normal RUNX2 protein. The proband was an 8-year-old girl without maxillary hypoplasia (see Figure 3B). Eruption of permanent teeth was delayed; however, typical supernumerary teeth were not evident (see Figure 3C and D). Instead, she had an additional left maxillary deciduous central incisor and a right mandibular deciduous central incisor. Additionally, the right mandibular central incisor was duplicated (see Figure 3B and C). The chest radiograph revealed complete absence of both clavicles (see Figure 3E).

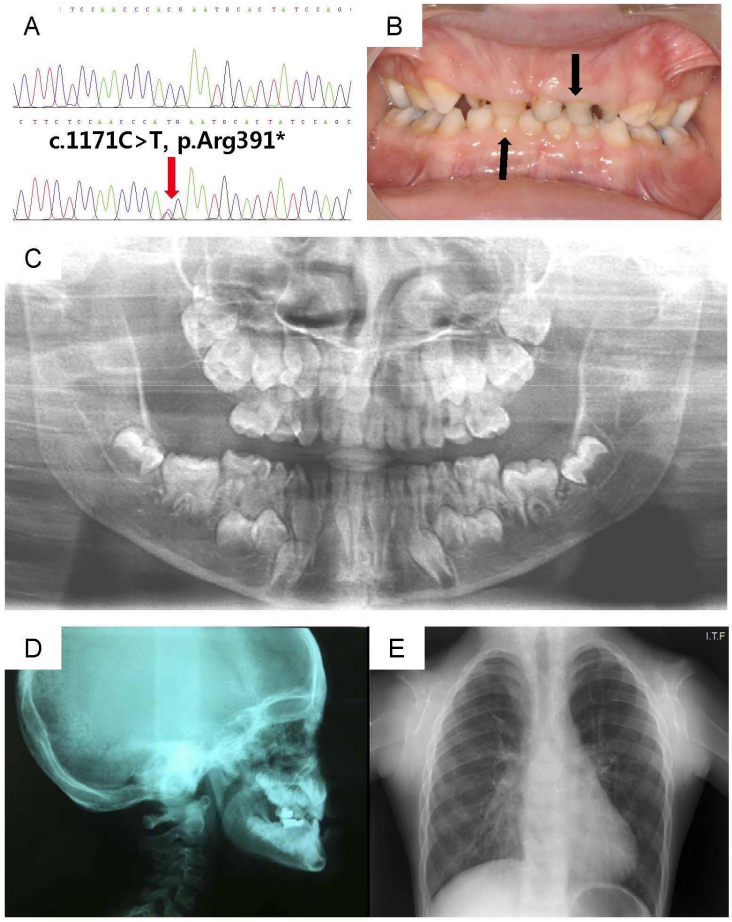

Figure 3. Mutational analysis, clinical photo, and radiologic examination of Patient 3. **A.** Mutational analysis reveals a single nucleotide deletion (c.1171C>T, p.Arg391*). **B.** Frontal clinical photo shows duplicated deciduous incisors (black arrows). **C.** Panoramic radiograph shows no typical supernumerary teeth, but duplicated mandibular left permanent incisor. **D.** Paranasal sinuses are hypoplastic, but maxilla is not hypoplastic. **E.** Both clavicles are aplastic.

DISCUSSION

The *RUNX2* gene has two distally located promoters, resulting in two major mRNA transcripts (Li and Xiao, 2007). The short transcript (NM_004348.3) was first cloned and controlled by the downstream promoter (Bae et al., 1993; Ogawa et al., 2003). This isoform (isoform c) encodes a 507-amino acid protein and starts its N-terminus with MRIPV (NP_004339.3). Subsequently, the upstream promoter was identified to control the expression of the following two transcripts. The first, the longest transcript (NM_001024630.3) encoding a 521-amino acid protein (NP_001019801.3), is the major product, and is a bone-specific isoform (isoform a) (Ducy et al., 1997; Stewart et al., 1997; Thirunavukkarasu et al., 1998).

The other transcript (NM_001015051.3) has an in-frame exon deletion in the coding region and encodes a 499-amino acid protein (NP_001015051.3, isoform b) (Geoffroy et al., 1998). The N-terminus of both isoforms, a and b, begins with MASNS.

All RUNX2 isoforms have common conserved domains. The Q/A domain is located in the N-terminus part of the protein and is composed of a polyglutamine stretch (23 amino acids) and a polyalanine stretch (17 amino acids). The length of these stretches is known to be important for the transcriptional activity of the RUNX2 protein (Mundlos et al., 1997; Thirunavukkarasu et al., 1998). The runt domain is a 128-amino acid motif, which interacts with CBFβ and binds to DNA sequences. This motif, originally identified in the *Drosophila* runt protein, is highly conserved in orthologous or paralogous proteins in humans or other vertebrates (Ahn et al., 1996; Eggers et al., 2002). The nuclear localization domain is located at the C-terminal end of the runt domain, and is important for the nuclear transport of this protein (Kanno et al., 1998). The C-terminal half of the RUNX2 protein is a proline-serine-threonine (PST)-rich domain, and the nuclear matrix targeting sequence (NMTS) locates in the middle of the PST domain (Zhang et al., 2000a; Lo Muzio et al., 2007). The NMTS domain determines the subnuclear localization of the RUNX2 protein and also has binding affinity with other proteins (Smads, p300, and histone deacetylase) (Zaidi et al., 2001; Afzal et al., 2005). The last five amino acids of the C-terminal end comprise the VWRPY motif with which TLE2 (a mammalian homolog of *Drosophila groucho*) interacts to inhibit *RUNX2* transcriptional activity (Aronson et al., 1997; Levanon et al., 1998).

In this study, we identified three mutations causing CCD. A missense mutation (c.674G>A, p.Arg225Gln) located in the runt domain is one of the mutational hotspots in RUNX2 (four arginine residues with CpG methylation) (Yoshida et al., 2002). The mutated protein was shown to bind to CBFβ, but not to DNA (Yoshida et al., 2002). The other two mutations observed were a frameshift mutation (c.1119delC, p.Arg374Glyfs*) and a nonsense mutation (c.1171C>T, p.Arg391*). The nonsense-mediated decay system (NMDS) can detect the presence of premature stop codons in the mRNA transcript and initiates degradation of the mRNA (Maquat, 2002; Moore, 2002). However, stop codons located in the last exon do not initiate NMDS and survive to be translated as a short, truncated protein (Wagner and Lykke-Andersen, 2002). Therefore, these two truncating mutations will lack half of the PST domain, including the NMTS and VWRPY domains.

Mutational studies have shown *RUNX2* mutations in approximately half of the CCD patients examined. Furthermore, heterozygous deletion and duplication of *RUNX2* genes were recently identified in CCD patients (Ott et al., 2010), and a large duplication downstream of *RUNX2* was also reported (Hansen et al., 2011), suggesting disruption of a possible regulatory site of *RUNX2*.

It has been suggested that mutations affecting the runt domain are associated with severe dental problems, such as multiple eruption failure and supernumerary teeth, while mutations outside of the runt domain show mild dental phenotypes (Bufalino et al., 2012). However, the genotype-phenotype correlation is weak, especially in terms of dental development alterations (Suda et al., 2007; Ryoo et al., 2010). Variable expressivity has been reported between families, and even among members of the same family (Zhang et al., 2010). Recently, RUNX2 haploinsufficiency resulting in excessive unbound Twist1, which enhances fibroblast growth factor signaling, has been reported as a molecular mechanism of supernumerary teeth formation in CCD patients (Lu et al., 2012). However, wide variation in the dental phenotype of CCD patients suggests that genetic modifiers and interacting partners await discovery.

In summary, we recruited three *de novo* CCD families and identified disease-causing mutations in *RUNX2*. Clinical examination revealed a unique dental phenotype in one patient, which has not been reported previously. We believe that this finding will broaden our understanding of the mechanism of supernumerary teeth formation and CCD-related phenotypes. Future studies on the molecular mechanism of supernumerary teeth formation related to *RUNX2* mutations may provide better insight into dental development.

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