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ORIGINAL ARTICLE

# *RUNX2* mutations in cleidocranial dysplasia patients

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**OBJECTIVE: Mutations in the *RUNX2* gene, a master regulator of bone formation, have been identified in cleidocranial dysplasia (CCD) patients. CCD is a rare autosomal-dominant disease characterized by the delayed closure of cranial sutures, defects in clavicle formation, and supernumerary teeth. The purposes of this study were to identify genetic causes of two CCD nuclear families and to report their clinical phenotypes.**
**MATERIALS AND METHODS: We identified two CCD nuclear families and performed mutational analyses to clarify the underlying molecular genetic etiology.**
**RESULTS: Mutational analysis revealed a novel nonsense mutation (c.273T>A, p.L93X) in family 1 and a *de novo* missense one (c.673C>T, p.R225W) in family 2. Individuals with a nonsense mutation showed maxillary hypoplasia, delayed eruption, multiple supernumerary teeth, and normal stature. In contrast, an individual with a *de novo* missense mutation in the Runt domain showed only one supernumerary tooth and short stature.**
**CONCLUSIONS: Mutational and phenotypic analyses showed that the severity of mutations on the skeletal system may not necessarily correlate with that of the disruption of tooth development.**
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**Keywords:** *RUNX2;* Cleidocranial dysplasia; mutation; supernumerary teeth

## Introduction

Investigations establish that heterozygous mutations in the *RUNX2* gene, the master regulator of bone formation, cause cleidocranial dysplasia (CCD, OMIM: 119600) (Lee *et al*, 1997; Mundlos *et al*, 1997). Haploinsufficiency of RUNX2 has long been believed to be a cause of CCD (Quack *et al*, 1999; Kim *et al*, 2006). CCD is a rare developmental bone disease that is characterized by the delayed closure of cranial sutures, aplasia or hypoplasia of clavicles, short stature, and dental anomalies such as supernumerary teeth and delayed eruption of permanent dentition (Mundlos, 1999).

The *RUNX2* gene has several isoforms with different transcription start sites and alternative splicing (Stock and Otto, 2005; Li and Xiao, 2007). Mutational and functional analyses provide valuable information for a better understanding of protein function as well as disease nature (Quack *et al*, 1999; Yoshida *et al*, 2002). Variations in expressivity make it difficult to find a mutational effect on both skeletogenesis and odontogenesis. Due to the absence of a standard nomenclature system for the *RUNX2* mutation, many reports use their own naming systems based on the specific isoform. This makes it even more difficult to deduce proper genotype–phenotype correlations.

In this study, we therefore aimed to identify underlying genetic defects in two Korean CCD families, and to report their clinical phenotypes with a review of transcriptional variants.

## Materials & methods

*Identification of kindreds and enrollment of human subjects*

This study was reviewed and approved by the Institutional Review Board at the Seoul National University Dental Hospital. Experiments were undertaken with the understanding and written consent of each subject in accordance with the Declaration of Helsinki. Both probands visited the department of pediatric dentistry for dental treatment without knowing that they had CCD. The father of the proband 1 was reported to be healthy, but to have supernumerary teeth. Both parents of the proband 2 had no known medical and dental problem. Radiographic and clinical examinations including palpation of the clavicles raised the possibility of CCD. Both probands and parents of the two families were included in the mutational analysis.

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*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 were determined by spectrophotometry from the $OD_{260}/OD_{280}$ ratio. We performed a mutational analysis of the exons and flanking intron sequences of the *RUNX2* gene for both families, as previously described (Zhang *et al*, 2000b), 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). Mutation numbering begins with the A of the second ATG start codon (starting MASNS) in exon 0 of a reference sequence (NM_001024630.3) as number 1.

## Results

*Mutational analyses*
*Family 1*. In our mutational analysis of the *RUNX2* gene, we found a single nucleotide change (c.273T > A) in exon 1 (Figure 1). This nucleotide change resulted in a nonsense mutation (p.L93X) between the polyglutamine/polyalanine (Q/A) repeat region and the Runt domain of the RUNX2 protein.

*Family 2*. Mutational analysis of the *RUNX2* gene revealed a missense mutation (c.673C > T, p.R225W) in the proband (Figure 2). We did not find this mutation in either the mother or the father of the proband.

*Clinical findings*
*Family 1*. The proband was a boy (11 years and 10 months old) with nine supernumerary teeth and maxillary hypoplasia. Radiographic examination revealed bilateral severely hypoplastic clavicles and hypoplasia of paranasal sinuses and mastoid air cells (Figure 1). His height (153 cm) was in the normal range (average: 151.8 cm for 12-year-old boys).

*Family 2*. The proband was a girl (10 years and 10 months old) with only one supernumerary tooth. Radiographic examination showed delayed closures of cranial sutures, hypoplasia of mastoid air cells and absence of clavicles (Figure 2). However, maxillary

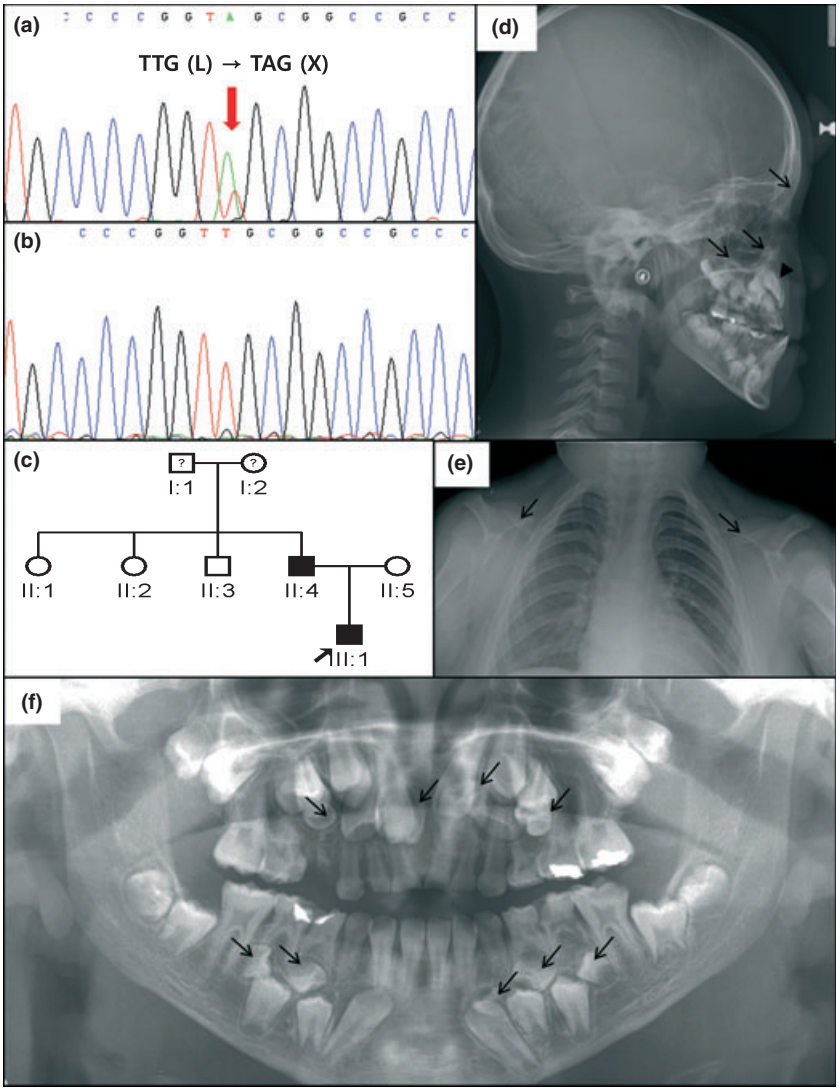


**Figure 1** Mutational analysis, pedigree, and radiographs of the family 1. (**a**) DNA sequencing chromatogram of an affected individual (proband) from family 1. The mutated nucleotide is indicated by a red arrow. (**b**) DNA sequencing chromatogram of unaffected individual. (**c**) Pedigree of the family 1. (**d**) Cephalometric radiograph showed hypoplasia of paranasal sinuses (black arrows) and hypoplastic maxilla (black arrowhead). (**e**) Chest radiograph showed hypoplastic clavicles (black arrows). (**f**) Panoramic radiograph revealed multiple supernumerary teeth (black arrows) and the delayed eruption of permanent teeth

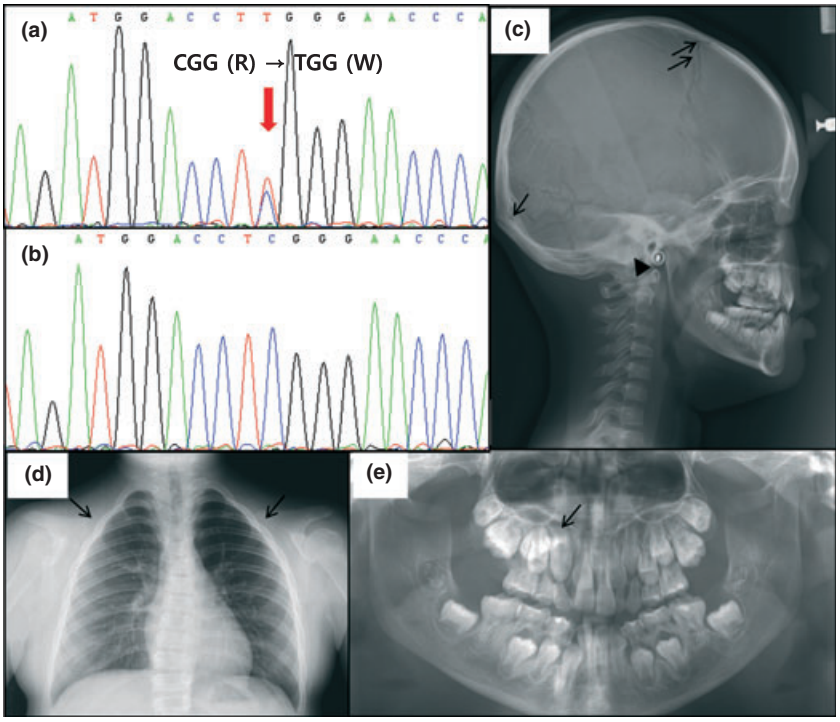


**Figure 2** Mutational analysis and radiographs of the family 2. (**a**) DNA sequencing chromatogram of an affected individual (proband) from family 2. The mutated nucleotide is indicated by a red arrow. (**b**) DNA sequencing chromatogram of unaffected individual. (**c**) Cephalometric radiograph revealed delayed closure of cranial sutures (black arrows) and hypoplasia of mastoid air cells (black arrowhead). (**d**) Chest radiograph showed aplastic clavicles (black arrows). (**e**) Panoramic radiograph revealed one supernumerary tooth in the right maxillary premolar region (black arrow)

hypoplasia and delayed eruption of permanent teeth were minimal. Her height (135 cm) was in the 5th percentile for her age group (average: 146.7 cm for 11-year-old girls).

## Discussion

The effect of the *de novo* mutation (c.673C > T, p.R225W) identified in this study was demonstrated previously, reportedly resulting in a loss of binding to DNA, but not to CBF$\beta$ (Yoshida *et al*, 2002). This mutation confirms the suggestion that the four arginine residues with CpG methylation are mutational hotspots (Yoshida *et al*, 2002). The novel mutation (c.273T > A, p.L93X) identified in this study introduced a premature stop codon, which may result in the degradation of mutant mRNA through a nonsense-mediated decay system (Shyu *et al*, 2008) or in the production of an extremely short protein with only the *N*-terminal part including an intact Q/A domain.

Runt-related transcription factor 2, *RUNX2*, is the master control gene of osteoblast differentiation (Ducy *et al*, 1997; Komori *et al*, 1997). Mutations in this gene are known to be responsible for the development of a congenital anomaly, cleidocranial dysplasia (CCD), in human (Mundlos *et al*, 1997) and mouse (Otto *et al*, 1997). According to the analysis of the protein structure, RUNX2 includes several functional domains (Figure 3): (i) the Runt domain is a 128 amino acid peptide motif originally described in the Drosophila runt protein, which is also highly conserved in ortholog proteins of other species or paralog proteins, such as RUNX1 or RUNX3, in human or other vertebrates (Ahn *et al*, 1996; Eggers *et al*, 2002). The Runt domain interacts with CBF$\beta$ and actively binds to highly conserved DNA sequences (Ogawa *et al*, 1993). Many mutations have been reported in this domain from CCD patients, indicating its importance in RUNX2 function (Quack *et al*, 1999; Yoshida *et al*, 2002). (ii) A nuclear localization signal (NLS) is located at the C-terminal end of the runt domain that is also highly conserved in many nuclear proteins and plays a critical role in the nuclear transportation of the protein (Kanno *et al*, 1998). (iii) A Q/A domain, a stretch of polyglutamine and polyalanine repeats, is located in the N-terminus of the protein. Based on the reports of a human mutation study (Mundlos *et al*, 1997) and an *in vitro* mutagenesis study (Thirunavukkarasu *et al*, 1998), the length of the Q or A stretch is important for the transcriptional activity of RUNX2. (iv) A proline–serine–threonine-rich (PST) domain comprises the C-terminal half of the protein in which these three amino acids are frequently identified. Deletion of this domain by the nonsense mutation or alternative splicing causes CCD by loss of function of the RUNX2 protein (Zhang *et al*, 2000a; Lo Muzio *et al*, 2007). (v) A VWRPY motif is located in the last five amino acids of the C-terminal end of all known runt-related proteins (Aronson *et al*, 1997). In Drosophila, VWRPY interacts with groucho and leads to the transcriptional repression of the runt. TLE2, a mammalian homolog of groucho, similarly interacts with the VWRPY domain and inhibits RUNX2 transcriptional activity (Levanon *et al*, 1998). No mutation has been reported in this domain. (vi) A nuclear matrix targeting sequence (NMTS) is a highly conserved 26 amino acid domain that determines subnuclear localization of the protein (Zaidi *et al*, 2001). This domain also has binding affinity with several important proteins such as Smads, p300 and histone deacetylase (Afzal *et al*, 2005).

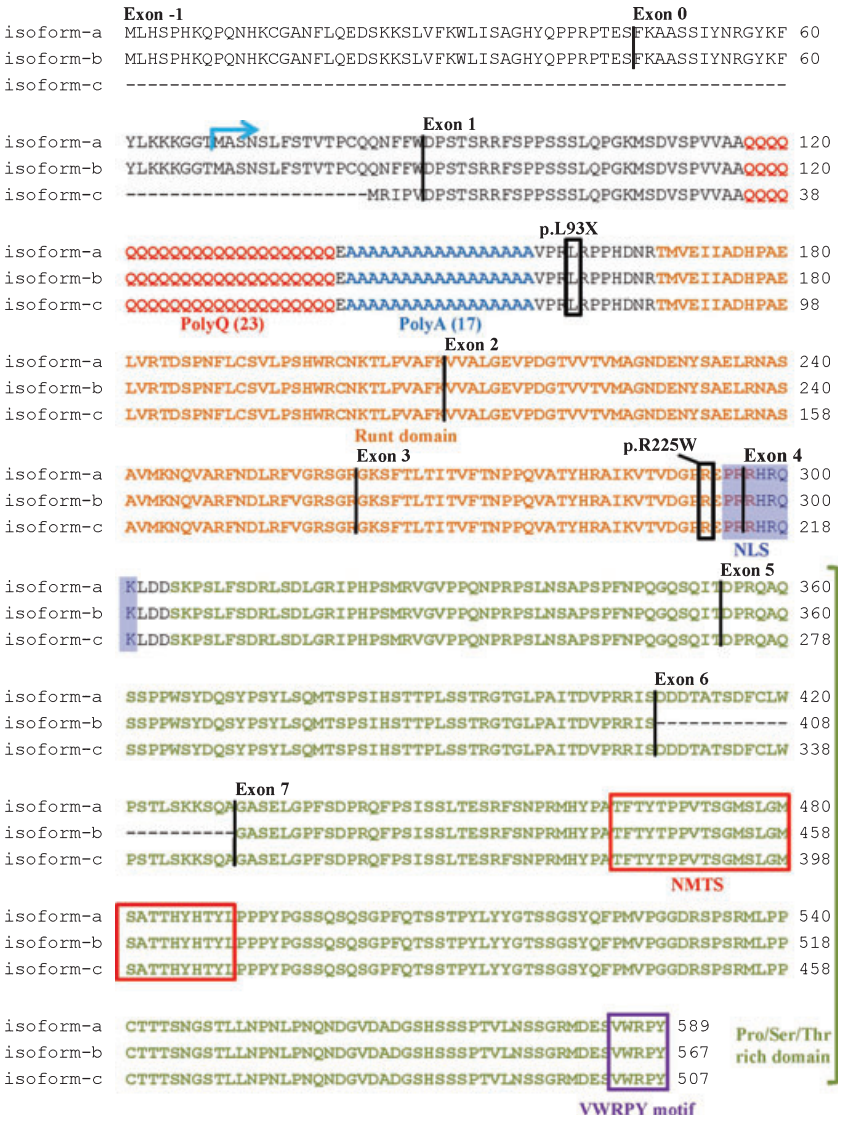


**Figure 3** Alignment of the amino acid sequence of human RUNX2 isoforms. The location of each exon was indicated from the first amino acid of each exon. Boundaries of exons are indicated by black vertical lines. The major translational product, bone-specific isoform, begins with MASNS in exon 0 (indicated with blue arrow). PolyQ and polyA domains are indicated by red and blue letters, respectively. The Runt domain is indicated by orange-colored letters. The nuclear localization signal (NLS) is indicated by the blue box. The proline–serine–threonine-rich (Pro/Ser/Thr) domain is shown in green letters. Nuclear matrix targeting sequence (NMTS) and VWRPY motif are indicated by red and purple boxes, respectively. The locations of the mutations identified in this study were indicated by black boxes

Genetic studies of CCD indicated that the *RUNX2* mutation has been identified in only about half of CCD patients, while the other half did not show any mutation with a causal relationship. In the middle of the mutation study, confusion developed due to different numbering systems of the RUNX2 amino acids. For example, the same mutation was designated as R179X (Zhang *et al*, 2000b) or R193X (Mundlos, 1999; Otto *et al*, 2002), which caused confusion for people who are not familiar with RUNX2. This confusion originates from the genomic structure of *RUNX2* gene (Figure 4). *RUNX2* has two distantly located promoters with two exons between the promoters leading to the production of two major mRNA transcripts with different 5′-UTR and 5′ coding sequences (Li and Xiao, 2007). Originally, RUNX2 type I (*RUNX2-I*) was first cloned and given the names *CBFA1*, *PEBP2aA*, or *AML3* (Bae *et al*, 1993; Ogawa *et al*, 1993). The *RUNX2-I* transcription is regulated by a downstream promoter to produce a 5.4 kb transcript that is translated into a 507 amino acid protein whose N-terminus starts with MRIPV (isoform-c,

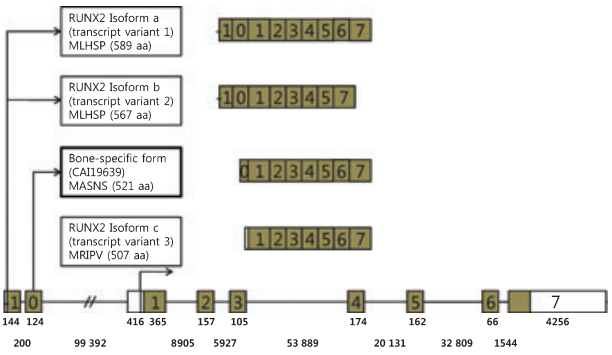


**Figure 4** Structure of human *RUNX2* gene and transcripts. The exons are represented as blocks numbered −1 to 7, and the introns are represented by lines. The number of nucleotides in each exon and intron is shown below the gene structure, and the transcript structures of each isoform are shown above the gene structure

NP_004339.3, NM_004348.3). More recently, many groups reported almost simultaneously that another upstream promoter controlling the expression of

*RUNX2-II*, a 6.2 kb transcript that is translated into 589 amino acid protein starting with MLHSP (isoform-a, NP_001019801.3, NM_001024630.3) (Ducy *et al*, 1997) or 521 amino acid protein with N-terminus starting with MASNS (CAI19639) (Stewart *et al*, 1997; Geoffroy *et al*, 1998). However, it has immediately identified that 589 amino acid protein has poor Kozak sequence (Thirunavukkarasu *et al*, 1998). Therefore, the 521 amino acid protein is the dominant protein translated from the *RUNX2-II* mRNA and is also known as an osteoblast specific isoform (Thirunavukkarasu *et al*, 1998; Sudhakar *et al*, 2001; Xiao *et al*, 2004). Additionally, there is an alternate splicing product (isoform-b, NP_001015051.3, NM_001015051.3) without exon 6 (Geoffroy *et al*, 1998). In this study, we used a RUNX2 mutation nomenclature using the longest transcript (NM_001024630.3), with the second ATG start codon (starting MASNS) in exon 0, instead of the first ATG start codon (starting MLHSP) in exon −1. This nomenclature can easily cover the alternatively spliced variants and also transcription start site of isoform-c (NP_004339.3, NM_004348.3) beginning with MRIPV also can be designated as c.59-16 (first nucleotide of exon 1 is 59th nucleotide if the A of the initiator ATG is taken as +1).

In this study, mutational analyses of CCD patients revealed *RUNX2* gene mutations. Phenotypic analysis showed that the severity of mutations on the skeletal system (formation of clavicles and stature) may not necessarily correlate with that of the disruption of tooth development. A standardized mutation nomenclature system with general consensus would help investigators collect and analyze reported mutations to understand the role of the *RUNX2* gene on the skeletal system, as well as dental development.

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## Author contributions

Hee-Yun Kang, Sook-Kyung Lee, and Kyung-Eun Lee performed experiments. Hyun-Mo Ryoo and Jung-Wook Kim designed the study and prepared the manuscript.

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