

***RUNX2* mutations in Taiwanese Patients with Cleidocranial Dysplasia**

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Abstract

Cleidocranial dysplasia (CCD) is an autosomal dominant human skeletal disorder comprising hypoplastic clavicles, wide cranial sutures, supernumerary teeth, short stature, and other skeletal abnormalities. It is known that mutations in the human *RUNX2* gene mapped at 6p21 are responsible for CCD. We analyzed the mutation patterns of the *RUNX2* gene by direct sequencing in six Taiwanese index cases with typical CCD. One of the patients was a familial case and the others were sporadic cases. Sequencing identified four mutations. Three were caused by single nucleotide substitutions, which created a nonsense (p.R391X), two were missense mutations (p.R190W, p.R225Q), and the fourth was a novel mutation (c.1119delC), a one-base deletion. Real time quantitative PCR adapted to determine copy numbers of the promoter, all exons and the 3'UTR region of the *RUNX2* gene detected the deletion of a single allele in a sporadic case. The results extend the spectrum of *RUNX2* mutations in CCD patients and indicate that complete deletions of the *RUNX2* gene should be considered in those CCD patients lacking a point mutation detected by direct sequencing.

Cleidocranial dysplasia, (CCD, MIM#119600), is an autosomal dominant skeletal dysplasia comprising defective skull ossification with frontal bossing, open fontanelles, clavicular hypoplasia, delayed ossification of the pelvis, late eruption of permanent teeth, malformed dental roots, supernumerary teeth, and normal intelligence (Mundlos, 1999).

Mutations in the core-binding factor A1 gene (*CBFA1*) located on chromosome 6p21 were identified as the cause of CCD (Gelb *et al.*, 1995; Mundlos *et al.*, 1997). This gene is composed of eight exons and spans a region of more than 220 kb in 6p21. The CBFA1 protein contains a highly conserved runt domain and therefore, the gene is also referred to as *RUNX2* (GenBank accession number AF001450) (Yoshida *et al.*, 2002). The runt domain interacts with the core binding factor consensus sequence TG^T/C GGT located in the promoter region of target genes. *RUNX2* regulates the expression of multiple genes expressed in osteoblasts and it is a cis-acting element in the osteocalcin promoter (Ducy *et al.*, 1997). *RUNX2* knockout mice show a complete lack of bone formation, whereas heterozygous *RUNX2* mutated mice display some of the hallmarks of human CCD, including open fontanelles and hypoplastic clavicles, but not the dental anomalies (Otto *et al.*, 1997).

The *RUNX2* protein contains two regions divided by the runt domain. On the N-terminal side of the runt domain are glutamine-alanine repeats (Q/A domain) and on the C-terminal side is a region rich in proline-serine-threonine (PST domain). The PST domain is thought to be the transactivation domain. The last five amino acids of *RUNX2* (VWRPY)

compose a conserved motif in all runt proteins (Quack *et al.*, 1999; Zhang *et al.*, 2000; Otto *et al.*, 2002).

Several mutations of *RUNX2* were identified in patients with CCD (Otto *et al.*, 2002). In this study, we screened six patients with typical CCD for mutations in the *RUNX2* gene.

Mutations were detected in five cases, these including an entire deletion of the *RUNX2* coding region.

The six unrelated patients included in this study were first evaluated because of short stature. They received the clinical diagnosis of CCD (for review of diagnostic criteria, see *Ref.*: Mundlos, 1999). Patient 4 was a familial case, whereas the others were sporadic. Except for Patients 5 and 6, their height was below the 5th centile for the Taiwanese population. All patients showed brachycephaly, mild frontal bossing, late tooth eruption with multiple crowded and supernumerary teeth, as well as clavicular hypoplasia. The upper part of the thorax was small and a funnel chest was noted. Scoliosis of the thoracic spine with convexity to the right and mild lordosis were also found. Chest radiographs showed hypoplasia of the clavicles with absence of the outer half. Three-dimensional head CT revealed enlarged anterior fontanelles beyond two years of age. Physical data for the six patients are summarized in Table 1.

Informed consent was obtained from the patients or the guardians of these patients.

Genomic DNA was extracted from peripheral blood leukocytes using a MagNA Pure LC

DNA Isolation Kit (Roche, Mannheim, Germany). The complete *RUNX2* coding region, including intron/exon boundaries, promoter region and 3'UTR were amplified according to Quack *et al.* (1999) and Napierala *et al.* (2005). PCR products were purified from the agarose gel using a QIAEX II kit (Qiagen, Hilden, Germany) and subjected to direct sequencing using a *BigDye* 3.1 Terminator cycle sequencing kit (Applied Biosystems, Forst City, CA, U.S.A.) with ABI Prism 377 DNA Sequencer or ABI 3100 Genetic Analyzer (Applied Biosystems, Forst City, CA, U.S.A.). To determine carrier rates for the novel mutations in the Taiwanese population, the *RUNX2* gene profile of 100 healthy individuals was analyzed using the same procedures. Reference sequence and base-pair numbers refer to GenBank accession numbers AF001443–AF001450 and NM_001024630.

RUNX2 mutations were detected in four patients. Patient 1 was heterozygous for a C-to-T transition at cDNA nucleotide position 1171 in exon 7 of *RUNX2*, this creating a stop codon at amino acid position 391 (p.R391X). The nonsense mutation, which caused early termination and produced a truncated protein lacking the PST-domain and the VWRPY motif (Thirunavukkarasu *et al.*, 1998), has been previously reported (Tsai *et al.*, 2000; Zhang *et al.*, 2000). The mutation seen in Patient 4 was in heterozygosis and represented a C-to-T transition at nucleotide 568 in exon 1 of *RUNX2*, this causing the substitution of arginine by tryptophan at amino acid position 190 (p.R190W) in the runt domain (Otto *et al.*, 2002). An arginine stretch located in the runt domain plays an important role in DNA binding. Therefore,

disruption of this region may lead to the loss of DNA binding ability (Yoshida *et al.*, 2002).

As the side chain of arginine is the major part that interacts with DNA, replacing it by another amino acids of different hydrophobicity and charge, such as tryptophan, disrupts the structure of the runt domain and decreases DNA binding affinity.

Patient 6 was heterozygous for a G-to-A transition at cDNA nucleotide position 674 in exon 3 of *RUNX2*, this causing arginine to be substituted by glutamine at amino acid position 225 (p.R225Q) at the C-terminal end of the runt domain (Otto *et al.*, 2002). This amino acid substitution abolishes the positive charge of the residue at this position and might affect the structure stability and interaction with other proteins.

In Patient 2, the cytosine at cDNA nucleotide position 1119 was deleted (c.1119delC), which led to a frameshift at amino acid 373, creating a stop codon at position 483 (Figure 1). This deletion has not been reported previously and was not present in the patient's parents. It disrupts both the repression subdomain and the VWRPY motif and is predicted to abolish *RUNX2* function. The C-terminal region of the PST-domain, which contains a region that interacts with Smad proteins, is considered necessary for *RUNX2*-mediated transcriptional regulation (Zhang *et al.*, 2000). The VWRPY motif is an interaction partner for the binding of transcriptional corepressors of the TLE/Grg family (Aronson *et al.*, 1997; Levanon *et al.*, 1998). Thus, even in cases where the runt domain is complete, the mutated *RUNX2* protein may fail to interact with other transcription factors (Thirunavukkarasu *et al.*, 1998; Zhang *et*

al., 2000).

In Patient 2, we furthermore found a single nucleotide polymorphism at cDNA nucleotide 240 (p.A80A), a substitution of G for A. This polymorphism site was described previously in another population (Machuca-Tzili *et al.*, 2002).

Patients 3 and 5 did not show any mutation in the promoter and the coding regions of *RUNX2*, similar to previous results showing that only 33~50% of patients with an unambiguous clinical and radiographic diagnosis of CCD carry mutations in the *RUNX2* gene (Quack *et al.*, 1999). Large deletions or duplication of the *RUNX2* gene are rarely seen in CCD patients (Lee *et al.*, 2008). Thus, to investigate the possibility of a gross *RUNX2* deletion, the genomic DNA of Patients 3 and 5 were subjected to real time quantitative PCR (RT-qPCR), following the analytical conditions described by Lee *et al.* (2008). Briefly, three independent experiments were performed to determine copy number variation in CCD patients and normal individuals. The RT-qPCR primers were designed to detect the promoter, exons, and 3'UTR regions of *RUNX2*. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as control. The formula for calculating copy number was: copy number = $2 * 2^{-(\Delta C_{tp} - \Delta C_{tn})}$ where Ct is the threshold cycle defined as the mean cycle at which the fluorescence curve reached an arbitrary threshold; ΔC_t is calculated as Ct of *RUNX2* - Ct of *GAPDH*, ΔC_{tp} is the ΔC_t for patients, and ΔC_{tn} that for normal individuals. While two copies of the tested *RUNX2* regions were present in Patient 5, Patient 3 had only one copy of

theses regions (Figure 2). Thus, we concluded that Patient 3 carried a deletion of one of the *RUNX2* alleles.

Although no mutation was found in Patient 5, following sequencing and RT-qPCR analysis, these results do not exclude other mutation types in the *RUNX2* gene, such as inversions, translocations, mutations within introns, etc. On the other hand, a report showed that cytogenetic anomalies at chromosomal band 8q22 have been associated with a CCD-like phenotype case (Brueton *et al.*, 1992). Thus, more detailed analyses are required to identify the genetic abnormality seen in Patient 5.

The patients with any of the four point mutations in the *RUNX2* gene were similar in their clinical symptoms, independent of whether their *RUNX2* protein retained the normal runt domain or not. The patients presented here lacking the PST domain and VWRPY motif exhibited typical CCD. This suggests that the C-terminal region plays an important role in *RUNX2* function, with different genotypes seemingly generating a similar CCD phenotype. From the findings of Patient 3, who carried the deletion of one allele, we conclude that a one-allele deletion affects the gene expression level and produces clinical symptoms similar to those of point mutations. We furthermore inferred that CCD patients not showing a *RUNX2* mutation in directed sequence data should be screened for a gene deletion or duplication, and RT-qPCR appears as a sensitive approach to this end.

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Table 1 - Summary of clinical manifestations and *RUNX2* gene mutation sites in five Taiwanese cases with cleidocranial dysplasia.

Case	Type	Age, years	Sex	Stature, cm (centile)	Clavicular hypoplasia	Dental anomalies	Open fontanelle	Mutation site on <i>RUNX2</i>	Note
1	Sporadic	4	M	91 (<3rd)	Y	Y	Y	c.1171 C→T, R391X	
2	Sporadic	11	M	127 (<5th)	Y	Y	Y	c.1119delC, frameshift	
3	Sporadic	2	F	78 (<3rd)	Y	Y	Y	exon 0~7, 3'UTR deletion	
4	Familial	10	M	122 (<5th)	Y	Y	Y	c.568 C→T, R190W	Child
		40	F	144 (<5th)	Y	Y	Y	c.568 C→T, R190W	Mother
5	Sporadic	12	M	141 (10~25th)	Y	Y	Y	N.D. ⁴	
6	Sporadic	8	F	127 (75th)	Y	Y	Y	c.674 G→A, R225Q	

Sex: M, male; F, female. Y: present; N: not present. N.D.: not detected.

Figure legends

Figure 1 - Sequence analyses of genomic DNA of the *RUNX2* from Patient 2. A heterozygous cytosine deletion at cDNA position 1119 was detected (c.1119delC). The normal sequence is shown above the electropherogram.

Figure 2 - Analysis of *RUNX2* copy number using RT-qPCR. Copy number was determined for the *RUNX2* promoter region, all of the exons and the 3'-UTR. Patient 3 had only one copy of these regions. ▲ : normal; ○: Patient 3; □: Patient 5. Each bar indicates mean \pm SD of three independent experiments.