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RESEARCH ARTICLE

Intragenic microdeletion of *RUNX2* is a novel mechanism for cleidocranial dysplasia

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Abstract Cleidocranial dysplasia (CCD; MIM 119600) is a rare autosomal dominant disorder characterized by facial, dental, and skeletal malformations. To date, rearrangement and mutations involving *RUNX2*, which encodes a transcription factor required for osteoblast differentiation on 6p21, has been the only known molecular etiology for CCD. However, only 70% patients were found to have point mutations, 13% large/contiguous deletion but the rest of 17% remains unknown. We ascertained a family consisted of eight affected individuals with CCD phenotypes. Direct sequencing analysis revealed no mutations in the *RUNX2*. Real time quantitative PCR were performed which revealed an exon 2 to exon 6 intragenic deletion in *RUNX2*. Our patients not only demonstrated a unique gene change as a novel mechanism for CCD, but also highlight the importance of considering "deletion" and "duplication" in suspected familial cases before extensive effort of gene hunting be carried.

Keywords Cleidocranial dysplasia · Intragenic deletion · Mechanism · RUNX2

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Introduction

Cleidocranial dysplasia (CCD; MIM 119600) is a rare autosomal dominant human skeletal disorder. The clinical features of CCD include facial and dental malformations characterized by delayed closure frontanelles, frontal bossing, absent clavicles, short stature, late eruption, and supernumerary permanent teeth and other skeletal anomalies (Mundlos 1999). There is considerable phenotypic variation for CCD, even within families (Chitayat et al. 1992). Mutations in the runt-related transcription factor 2 gene (*RUNX2*, also known as *CBFA1*, *PEBP2αA*, and *AML3*) located on chromosome 6p21 (Mundlos et al. 1997) have been identified as the cause of CCD. *RUNX2* is one of the three mammalian homologs of the Drosophila *runt* gene, which encodes a transcription factor required for osteoblast differentiation. *RUNX2* spans a region over 220 kb in 6p21 and is composed of eight exons and several splice variants have been described (Geoffroy et al. 1998). It has also been reported that *RUNX2* is transcribed from two promoters (the distal promoter P1 and the proximal promoter P2) (Stewart et al. 1997). Numerous mutations in

RUNX2 have been identified in patients with CCD (Otto et al. 2002; Yoshida et al. 2002; Zhou et al. 1999). Most of the missense mutations were located in the runt region (Baumert et al. 2005; Otto et al. 2002; Yoshida et al. 2002) involving heterodimerization and DNA binding with CBFβ. This discrepancy in distribution could be explained by that the runt domain is highly conserved and is less resistant to single nucleotide changes. Nonsense, splicing mutation, and insertion/deletions were also found and they were scattered throughout the entire *RUNX2* gene. Deletion of the entire *RUNX2* gene or larger has been described (Mundlos et al. 1995, 1997; Otto et al. 2002; Quack et al. 1999) and in one case the deletion spanning both *RUNX2* and its upstream *VEGF* gene with the patient exhibiting both CCD and cardiovascular defects (Izumi et al. 2006).

Numerous CCD patients without any detectable mutations in *RUNX2* by sequencing or FISH have been identified (Kim et al. 2006; Otto et al. 2002; Quack et al. 1999; Yoshida et al. 2002). This would indicate a genetic heterogeneity such as mutation in RUNX2 gene's interacting proteins or regulatory elements or due to other mechanism that was not yet reported. One recent study identified a case with *CBFβ* mutation which encodes an interacting molecule of RUNX2. This individual did not have classical CCD phenotypes but exhibited delayed skull ossification and cleft palate (Khan et al. 2006).

In this study, we ascertained an extended family with many have classic yet severe CCD phenotypes. However, sequencing analysis did not reveal any mutations in *RUNX2* and the results of FISH study were not confirmative. Further analysis using real time PCR, Southern blot, and reverse PCR revealed a novel microdeletion of about 125.6 kb and defined the breakage points in one allele of the gene. While intra-gene deletion involving multiple exons has been reported in many other genes, it has not been reported in CCD. The molecular mechanism for such deletion and the characteristic phenotype in this family are also discussed.

Materials and methods

Patients

The extended CCD family was ascertained in a tertiary medical center. Proband III: 3 (pedigree see Fig. 1) was initially evaluated for hypertelorism and developmental delay; however, was later excluded to suffer from CCD. Given the provided family history of CCD, 28 out of the 47 traceable extended family members were recruited for this study. Clinical evaluations were performed on all the participants for typical signs of CCD including radiographs to detect abnormalities in clavicles, skulls, and hand. About 10 ml of blood was drawn for DNA extraction. About 10 ml of blood was also drawn from three individuals (two normal individuals and one patient) for cell line transformation used for FISH analysis. This project has been approved by the Institution Review Board of Academia Sinica and China Medical University. Informed consent was obtained from every participating individual.

Real time quantitative PCR (qPCR) for copy number analysis

RUNX2 copy number was determined by real time quantitative PCR reactions performed using Power SYBR GREEN PCR Master kit (Applied Biosystems, Foster City, CA, USA). Three independent experiments were performed to determine the variation in copy number between CCD patients and normal individuals with duplicate samples for each experiment. The RT-qPCR primers were designed according to manufacturer's instruction. Primers were designed to detect copy number of the promoter, exons, and 3′ UTR regions of *RUNX2*. The qPCR reactions were performed using the ABI Prism® 7900HT Sequence Detection system and the fluorescent signal intensity was recorded on ABI Prism 7900HT Sequence Detection system and analyzed by Sequence Detector v2.3 software.

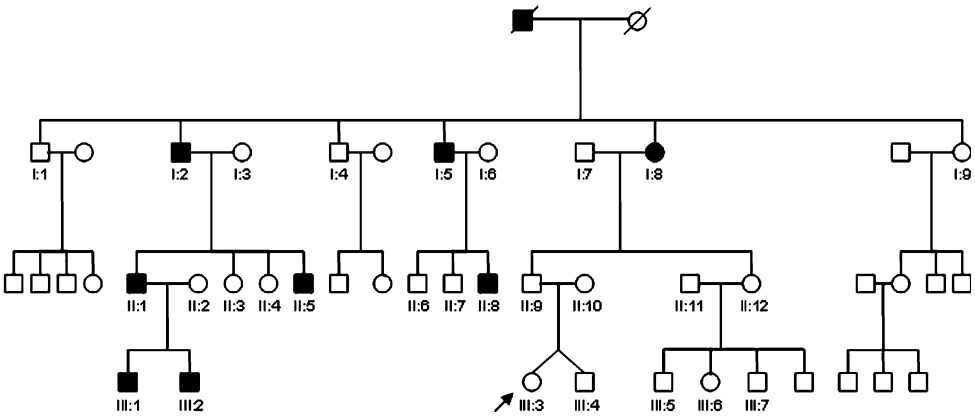

Fig. 1 Pedigree of the extended family with CCD phenotypes. The 28 subjects recruited in this study are numbered. The arrow indicates the proband

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. The formula for calculating copy number was: copy number $= 2 * 2^{-(\Delta Ctp - \Delta Ctn)}$ where Ct was the threshold cycle defined as the mean cycle at which the fluorescence curve reached an arbitrary threshold; ΔCt was calculated as Ct of RUNX2 − Ct of GAPDH, ΔCtp was the ΔCt of patients, and ΔCtn was the ΔCt of normal individuals. Two normal individuals were used in the experiments.

Inverse PCR for deletion mapping

Inverse PCR is a method for the rapid *in vitro* amplification of unknown DNA sequences that is flanked by a region of known sequences (Ochman et al. 1988). Restriction digestion was first carried out as described above and the enzymes were inactivated at 65°C for 20 min. The digested DNA fragments were allowed for self-ligation to generate circular DNA. Amplification was then performed with outward facing primers 5′-GTTCCTGCAAAGAATGGTCC-3′ and 5′-TAGAGCAGGGAAACCCACAG-3′. Sequencing of the unknown region can then be performed on the amplified DNA with the above primers.

Results

Clinical data

Of the 28 individuals recruited for this study, 8 were confirmed with CCD (Fig. 1). The eight affected individuals in the CCD family all had delayed closure frontanelles, frontal bossing, clavicles hypoplasia, dental anomalies, and short stature. The average adult height was 137 cm (<2 percentile) for the adult female CCD patient and 150 cm (<5 percentile) for the adult male CCD patients. Age of the patients ranged from 6 to 67 years old. No other medical conditions were present other than the presence of osteoarthritis in the elderly patients (I:5 and I:8). In addition to the typical CCD phenotypes, the patients also exhibit unusual CCD phenotypes such as hypoplasia in the distal phalanges and all middle phalanges have cone-shaped epiphyses (Fig. 2).

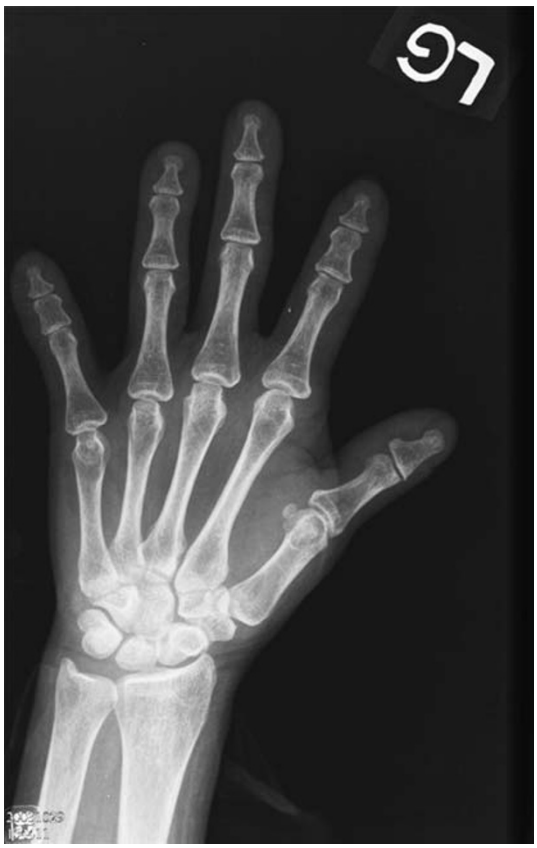

Fig. 2 Radiograph of patient I:8 showing phalange abnormalities

Molecular analysis of the RUNX2 gene

Initial sequencing of all exons, intro-exon junctions, and 2 kb upstream of transcription start site of the *RUNX2* failed to identify any mutations in the gene. FISH analysis was subsequently performed on one patient (I:8) and one normal control in this extended family to determine the presence of deletions in RUNX2. The signals on one of the chromosome 6 homologs in the patient's cells appeared weakened (data not shown). These findings suggested that one of the chromosome 6 of the CCD patient could have deletion involving a portion of the *RUNX2* gene.

Real time quantitative PCR (RT-qPCR) was performed to determine the copy number of each of the exons and the 3′UTR of *RUNX2*. The qPCR results of eight CCD patients and two normal individuals in this extended three generation family revealed that *RUNX2* was deleted from exon 2 to exon 6 in all eight patients as indicated by the copy number of one while the normal individuals had the normal copy number of two (Fig. 3). This demonstrated that the intragenic deletion was the cause of CCD in this family.

Southern blot analysis was next performed to narrow down the region that harbored the 3′ break point and mapped the 3′ breakage point to within 1 kb between exon 6 and exon 6.1 (data not shown). This also confirmed the qPCR data that the deletion was indeed present.

Inverse PCR was then performed on the restriction digest fragments and the resulting circular DNAs were sequenced (data not shown). By comparing the sequence from inverse PCR to the reference sequence of *RUNX2*, the break points of the deletion were identified (Fig. 4a). A total of 125.6 kb was deleted, spanning intron 1 (IVS + 77447) to intron 6 (IVS6 + 19466) with both ends of

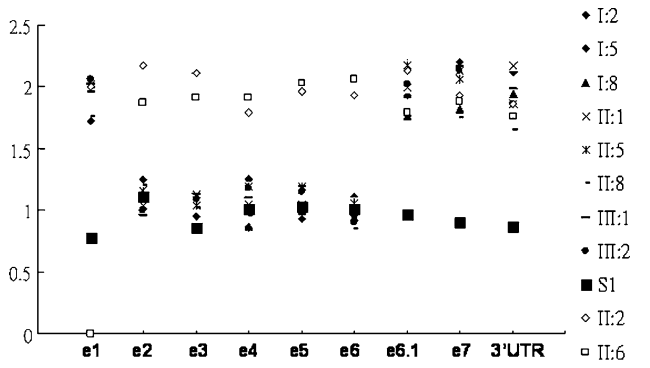

Fig. 3 Analysis of RUNX2 copy number using RT-qPCR. Copy number of RUNX2 was determined for all the exons and 3′UTR. I:2, I:5, I:8, II:1, II:5, II:8, III:1, III:2 were patients and II:2 and II:6 were controls from the CCD extended family. S1 was a sporadic case

breakage points containing ATC (Fig. 4). Sequence of the break points was also confirmed by direct sequencing.

Discussion

The work presented here described the first large intragenic microdeletion: exons 2–6 were deleted in the three generation CCD family. This deletion created a truncated protein without most of the N-terminal domain. Without the DNA binding runt domain, this protein was unable to modulate transcription of RUNX2 downstream genes. Consequently, CCD phenotype arose as a result of haploinsufficiency of RUNX2 (Mundlos et al. 1997; Otto et al. 1997).

This family was significantly shorter than the reported cases (137 vs 156) and (150 vs 165) (Cooper et al. 2001). The average height of the affected adults was 148 cm. While we cannot exclude the contribution of ethnic background for the shorter stature, the short distal phalangeal hyperplasia and small hands were very significant in this family and could be explained by this intragenic deletion. Cesarean section has been reported to be unusually high up to 69% (Cooper et al. 2001); however, it is not reported in this family.

Using Southern blot and inverse PCR, we have determined precisely the breakage points of the *RUNX2* deletion in this three generation CCD family. It is interesting to note that both ends contain the same three nucleotides ATC. However, the sequence homology is probably too short for homologous recombination to occur. Thus, this particular intragenic deletion is most likely generated through non-homologous end joining.

FISH is a useful tool to detect microdeletion; however, its sensitivity depends on the size of the microdeletion and the location and size of the probe. The BAC clone used, RP11-1019C24 (191 kb), located within the *RUNX2* gene (220 kb) should be the best probe for the detection of *RUNX2* deletions. However, even with this probe the FISH study was inconclusive due to the partial *RUNX2* deletion.

The work described here can also be a good example for other studies. While genetic heterogeneity and pathway molecules can be the alternative mechanism when a mutation is not found, we suggest detailed study for a known gene before ever-ending effort in linkage be put forward as a general rule. When sequence variants are not detected by direct sequencing, real time PCR assays similar to the one used in this study or MLPA would allow detection of gene deletion or duplication efficiently.

In summary, we have identified the first intragenic microdeletion in *RUNX2* in a CCD family. Current clinical testing by sequence-based study only detects 60–70% of individuals with a clinical diagnosis of CCD. Microdeletion

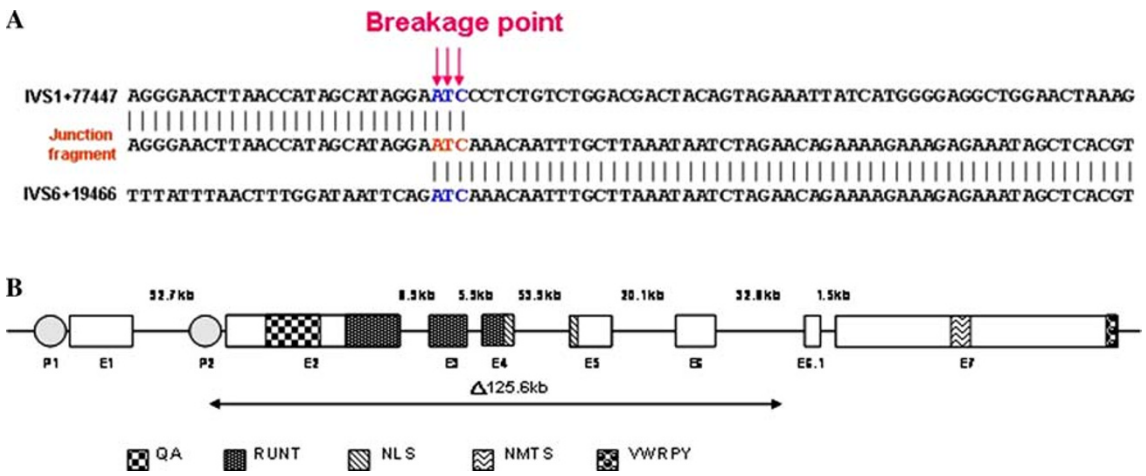

Fig. 4 Mapping of the RUNX2 deletion in the CCD family. (**a**) Sequencing of the inverse PCR products revealed the exact breakage point which is indicated by the arrows. The top and the bottom lines represent the sequences close to the break point in introns 1 and 6, respectively. (**b**) Schematic of the deletion which is 125.6 kb long and encodes most of the important functional domains of RUNX2

with contiguous deletion has been suggested to account for another 13% (Mendoza-Londono and Lee 2008). In our cohort, 28% is due to deletion (unpublished data). Our patients demonstrated a rare and novel deletion for CCD. We therefore suggest that in patients whose mutation is not found by traditional sequencing, the deletion/duplication assay, either RT-qPCR/MLPA, needs to be done particularly in a disease haploid insufficiency is thought to be the main cause. The deletion/duplication assay can improve the molecular diagnosis of CCD and likely change the statistics of molecular mechanism of this disease.

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References

Baumert U, Golan I, Redlich M, Aknin JJ, Muessig D (2005) Cleidocranial dysplasia: molecular genetic analysis and phenotypic-based description of a Middle European patient group. Am J Med Genet A 139(2):78–85. doi:10.1002/ajmg.a.30927

Chitayat D, Hodgkinson KA, Azouz EM (1992) Intrafamilial variability in cleidocranial dysplasia: a three generation family. Am J Med Genet 42(3):298–303. doi:10.1002/ajmg.1320420307

Cooper SC, Flaitz CM, Johnston DA, Lee B, Hecht JT (2001) A natural history of cleidocranial dysplasia. Am J Med Genet 104(1):1–6. doi:10.1002/ajmg.10024

Geoffroy V, Corral DA, Zhou L, Lee B, Karsenty G (1998) Genomic organization, expression of the human CBFA1 gene, and evidence for an alternative splicing event affecting protein function. Mamm Genome 9(1):54–57. doi:10.1007/s003359900679

Izumi K, Yahagi N, Fujii Y, Higuchi M, Kosaki R, Naito Y et al (2006) Cleidocranial dysplasia plus vascular anomalies with 6p21.2 microdeletion spanning RUNX2 and VEGF. Am J Med Genet A 140(4):398–401. doi:10.1002/ajmg.a.31061

Khan A, Hyde RK, Dutra A, Mohide P, Liu P (2006) Core binding factor beta (CBFB) haploinsufficiency due to an interstitial deletion at 16q21q22 resulting in delayed cranial ossification, cleft palate, congenital heart anomalies, and feeding difficulties but favorable outcome. Am J Med Genet A 140(21):2349–2354. doi:10.1002/ajmg.a.31479

Kim HJ, Nam SH, Kim HJ, Park HS, Ryoo HM, Kim SY et al (2006) Four novel RUNX2 mutations including a splice donor site result in the cleidocranial dysplasia phenotype. J Cell Physiol 207(1):114–122. doi:10.1002/jcp. 20552

Mendoza-Londono R, Lee B (2008) Cleidocranial dysplasia. Gene Rev. Available online http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=CCD

Mundlos S (1999) Cleidocranial dysplasia: clinical and molecular genetics. J Med Genet 36(3):177–182

Mundlos S, Mulliken JB, Abramson DL, Warman ML, Knoll JH, Olsen BR (1995) Genetic mapping of cleidocranial dysplasia and evidence of a microdeletion in one family. Hum Mol Genet 4(1):71–75

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S et al (1997) Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89(5):773–779. doi:10.1016/S0092-8674(00)80260-3

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120(3):621–623

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR et al (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89(5):765–771. doi:10.1016/S0092-8674(00)80259-7

Otto F, Kanegane H, Mundlos S (2002) Mutations in the RUNX2 gene in patients with cleidocranial dysplasia. Hum Mutat 19(3):209–216. doi:10.1002/humu.10043

Quack I, Vonderstrass B, Stock M, Aylsworth AS, Becker A, Brueton L et al (1999) Mutation analysis of core binding factor A1 in patients with cleidocranial dysplasia. Am J Hum Genet 65(5):1268–1278. doi:10.1086/302622

Stewart M, Terry A, Hu M, O'Hara M, Blyth K, Baxter E et al (1997) Proviral insertions induce the expression of bone-specific isoforms of PEBP2alphaA (CBFA1): evidence for a new myc collaborating oncogene. Proc Natl Acad Sci USA 94(16):8646–8651. doi:10.1073/pnas.94.16.8646

Yoshida T, Kanegane H, Osato M, Yanagida M, Miyawaki T, Ito Y et al (2002) Functional analysis of RUNX2 mutations in Japanese patients with cleidocranial dysplasia demonstrates novel genotype–phenotype correlations. Am J Hum Genet 71(4):724–738. doi:10.1086/342717

Zhou G, Chen Y, Zhou L, Thirunavukkarasu K, Hecht J, Chitayat D et al (1999) CBFA1 mutation analysis and functional correlation with phenotypic variability in cleidocranial dysplasia. Hum Mol Genet 8(12):2311–2316. doi:10.1093/hmg/8.12.2311