

RUNX2 Quadruplication: Additional Evidence Toward a New Form of Syndromic Craniosynostosis

Matthew R. Greives, MD,* Eric A. Odessey, MD,* Darrel J. Waggoner, MD,†‡ Deana S. Shenaq, MD,* Swaroop Aradhya, PhD,§ Allison Mitchell, MS, CGC,§ Emma Whitcomb, MD,¶|| Neil Warshawsky, DDS, MS,|| Tong-Chuan He, MD, PhD,* and Russell R. Reid, MD, PhD*‡

Abstract: The RUNX2 transcription factor regulates osteoblast differentiation. Its absence, as with cleidocranial dysplasia, results in deficient bone formation. However, its excess seems to follow a dose response of over ossification. RUNX2 duplications (3 copies) are exceedingly rare but have been reported to cause craniosynostosis. There are no existing reports of quadruplications (4 copies). We present a case study of a boy with an atypical skull deformity with pan-craniosynostosis whose microarray analysis revealed 4 copies of a 1.24-Mb region from 6p12.3 to 6p21.1 containing the RUNX2 gene. Further characterization of this osteogenic pathway may aid in our understanding of the pathogenesis and subsequent prevention and treatment of syndromic craniosynostosis.

Key Words: RunX2, cleidocranial dysplasia, craniosynostosis

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RUNX2 is a gene located at chromosomal segment 6p21 and has been studied extensively because of its regulatory effect on both endochondral and intramembranous bone formation.¹ Specifically, the RUNX2 protein coordinates multiple signaling pathways that regulate osteoblast differentiation and function.² Its activity is modulated by a variety of factors, such as fibroblast growth factor 2 (FGF-2), bone morphogenetic proteins (BMPs), parathyroid hormone, growth hormone, and mechanical loading. RUNX2 regulates bone formation via regulation of osteoblast-specific genes, such as osteocalcin, alkaline phosphatase, collagenase-3, bone sialoprotein, and collagen type I.^{1–7}

A variety of RUNX2 mutations have been reported, including chromosomal translocations, deletions, insertions, nonsense and splice-site mutations, and missense mutations.^{1,8–12} All of these mutations result in loss of function, leading to only 1 functional copy of the gene and subsequent deficient bone formation in a syndrome

known as cleidocranial dysplasia (CCD). Phenotypically, CCD is characterized by hypoplastic or aplastic clavicles, delayed cranial suture closure, and abnormal dentition.¹³

There have also been rare reports of 6p21 duplications, providing 3 copies of RUNX2, including one familial case.¹⁴ In 1 case, the resulting phenotype included dolichocephaly, downslanted palpebral fissures, wide nasal bridge, beaked nose, microretrognathia, and malformed ears with premature closure of the fontanelles by 7 months and premature death at age 16 months. There were no imaging studies taken to clearly define the morphology of the cranial sutures.¹⁵ Of the remainder of reported cases,^{16–18} only one links multiple copies of 6p21 specifically with craniosynostosis.¹⁹

We report a pediatric case with a 6p quadruplication resulting in 4 functional copies of RUNX2. The phenotypic manifestations of this new genetic syndrome follow logically from a presumed gain in osteoblast differentiation.

METHODS

This case was exempt from review per the guidelines of the University of Chicago institutional review board.

Gene Microarray

A half microgram each of patient DNA and sex-matched reference DNA was labeled with Cy5 and Cy3, respectively, using a commercially available random primer labeling kit (Enzo). The labeled DNA samples were purified on a QiaQuick purification kit (Qiagen), combined, and hybridized to a custom-designed 44K oligonucleotide array (Agilent Technologies, Santa Clara, CA) at 65°C for 40 hours. The arrays were washed in commercially available solutions, wash buffers I and II, and scanned on an Agilent scanner. The resulting TIFF images were analyzed with Feature Extraction software, and output from this software was imported into CGH Analytics software for final analysis. The data were analyzed using a z-score algorithm with a 0.25 cutoff threshold. The wash buffers, scanner, and all software were available from Agilent Technologies, Santa Clara, CA.

CLINICAL REPORT

Clinical History and Findings

C.H. presented at an outside hospital as an infant with airway obstruction from choanal atresia. He was diagnosed with a closed anterior fontanelle, ventricular septal defect, restricted range of motion in his elbows, mild conductive hearing loss, and developmental delays. He was treated with a tracheostomy until 1 year of age as well as hearing aids. There was no family history of craniofacial dysmorphism.

He was first examined at our institution at age 10. Craniofacial examination revealed an atypical skull deformity with pan craniosynostosis, specifically with temporal bulging bilaterally and high

From the *Department of Surgery, and †Genetics; ‡Pediatrics, Comer Children's Hospital, University of Chicago Medical Center, Chicago, IL; §GeneDx, Gaithersburg, MD; ||GetItStraight Orthodontics, and ¶Department of Pathology, University of Chicago Hospital, Chicago, IL.

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Address correspondence and reprint requests to Dr. Russell R. Reid, Section of Plastic Surgery, 5841 S. Maryland Ave, M/C 6035, Chicago, IL 60637; E-mail: rreid@surgery.bsd.uchicago.edu

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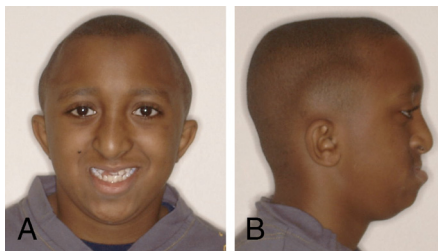


FIGURE 1. Frontal (A) and right (B) lateral views of the patient. Note the atypical skull deformity, exorbitism, and severe midface retrusion.

temporal grooving but no obvious sutural ridging. The forehead seemed mildly bossed, and there was mild supraorbital ridge retrusion. Orbital examination demonstrated mild-to-moderate exorbitism with adequate palpebral coverage bilaterally. The mean intercanthal distance was 26 mm. Midfacial examination revealed severe midfacial and malar retrusion from the inferior orbital rims down through the piriform aperture (Figs. 1A, B). Intraoral examination demonstrated severe class III malocclusion with a negative overjet that approximated 12 mm. The dentition was poor with multiple unerupted permanent teeth (Fig. 2A). This was confirmed on the panorex examination (Figs. 2B, C). Computed tomography revealed sagittal, squamosal, and partial lambdoid synostosis (Figs. 3A–C).

In terms of extracranial findings, C.H. was evaluated by the pediatric orthopedic team for complaints of worsening elbow stiffness and severe limitations in pronation and supination. Plain film radiography revealed intact bony structures but bilateral radial head dislocations (Figs. 4A, B).

Initial routine genetic screening included sequence analysis of exons 8 and 10 of *FGFR2* and the *TWIST* gene and mutational analysis for the Pro250Arg mutation in the *FGFR3* gene and the Pro252Arg mutation in the *FGFR1* gene. The results of these tests were negative. A whole genome comparative 44,000 oligonucleotide GenomeDx microarray v1 was then performed and demonstrated a quadruplication of a 1.24 Mb region on the short arm of chromosome 6 from 6p12.3 to 6p21.1 (Fig. 5). This region contains the *RUNX2* gene.

Operative Plan and Therapy

Given his clinical findings, we recommended that he undergo Lefort III distraction or wait for skeletal maturity and complete a Lefort I advancement with bone grafting. However, because of his complicated medical history and social situation, an extended workup delayed immediate surgical intervention. Presurgical orthodontic therapy was initiated. Because of the severity of the craniofacial dysmorphism, severe class III malocclusion, and psychosocial issues at school, a LeFort I halo distraction was performed at the age of 12. Given the self-consciousness typical of this age

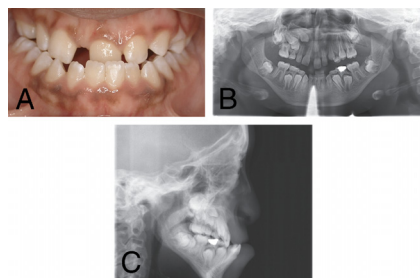


FIGURE 2. View of the patient's disorganized dentition (A), Panorex of dentition (B), lateral cephalogram and (C) demonstrating malocclusion.

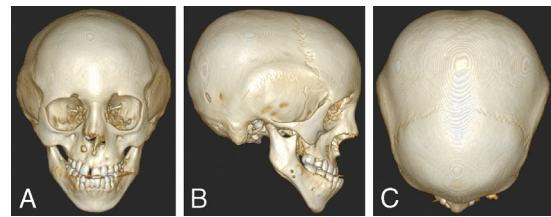


FIGURE 3. Frontal (A), right lateral (B), and vertex (C) views of the 3-dimensional CT, revealing sagittal, squamosal, and partial lambdoid synostosis.

group, we elected to use a rapid distraction protocol²⁰ to minimize halo wear time. After a 48-hour latency period, activation was implemented with 1-mm advancement twice daily for 7 days, followed by 1 mm per day for an additional 7 days. The final midface advancement was 21 mm over the 2 weeks. Per protocol, the patient returned to the operating room 12 days into the consolidation phase, to undergo device removal and ORIF of the maxilla using a pre-fabricated acrylic occlusal splint. Upon surgical exploration of the osteomy sites, however, we found premature ossification where typically a primordial, fibrous callus is present within the regenerate. This area was biopsied and found to contain mature bone without evidence of primordial callus (Figs. 6A, B). Because of the stability of the maxilla in its new position, we elected to not place rigid fixation. The patient was instead placed in Class II elastics to guide his occlusion. Two weeks of strict elastics were followed by 2 weeks of guiding (intermittent) elastic therapy. Postoperative evaluation up to 3 months demonstrates a stable occlusion without evidence of relapse. C.H. is currently in the postsurgical orthodontic phase of therapy to improve his occlusion. His 18-month follow-up images show significant improvement in his overall occlusion. (Fig. 7)

In regard to his upper extremities, C.H. had severely limited motion of his right arm, with flexion and extension of his right elbow between 70 and 90 degrees. Under general anesthesia, his right radius was ranged with improvements in extension to 25 degrees and flexion to 135 degrees. Unfortunately, he did not comply with his physical therapy and underwent a second ranging procedure, at the time of external distractor removal, by the orthopedic surgery team. His radial head dislocations were unable to be adequately reduced either time, although his functional status of his elbow joint was greatly improved.

DISCUSSION

Loss-of-function mutations of the *RUNX2* gene are associated uniformly with cleidocranial dysplasia, which is marked primarily by lack of bone development within the cranial sutures and clavicles. This disease is a direct result of the downregulation of osteoblast differentiation because of the loss of function of *RUNX2*. Although

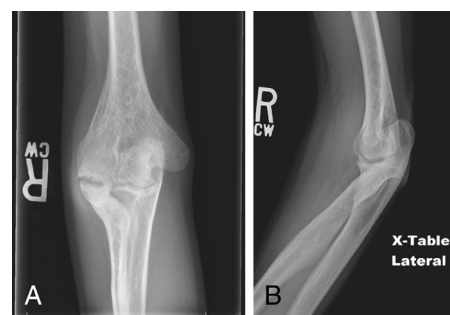


FIGURE 4. Right elbow radiograph (A and B) demonstrating dislocated radial head.

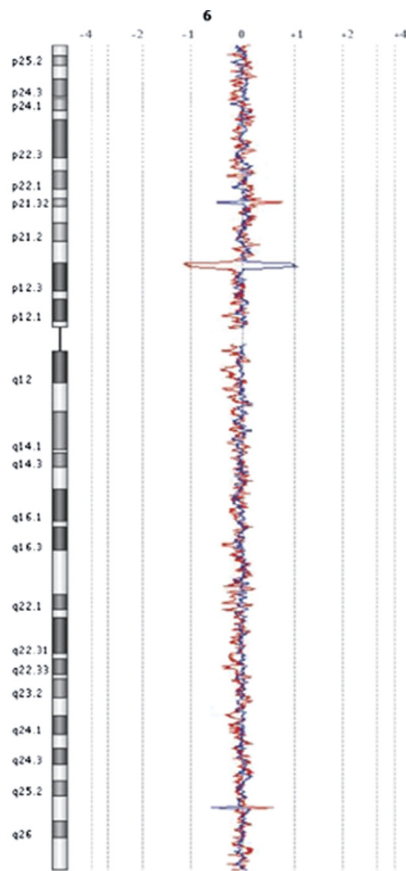


FIGURE 5. Results of oligonucleotide array CGH for chromosome 6 showing the duplicated region at 6p12.3 to 6p21.1.

many mutations of the *RUNX2* gene have been described, all share in common the result of heterozygous loss of protein function.

In cleidocranial dysplasia, this loss of function results in delayed cranial suture closure, with patent fontanelles persisting into adolescence. In severely affected individuals, clavicles may be absent, allowing the patients to touch their shoulders at the midline. Other associated traits include cleft palate and supernumerary permanent teeth.

It would logically follow, therefore, that a gain in function of the *RUNX2* protein would lead to many of the opposite traits as CCD. Scattered case reports of *RUNX2* duplications (3 copies) confirm this hypothesis. Williams et al¹⁹, for example, described a 6-year-old patient with a 6p21 duplication, who presented with craniofacial dysmorphism related to unicoronal and sagittal craniosynostosis, as well as maxillary hypoplasia, generalized hypotonia, and moderate mental deficiency. Mefford et al¹⁴ present 2 first cousins with a 6p21 duplication who presented with metopic craniosynostosis, abnormal dentition, although only one of them had motor delay.

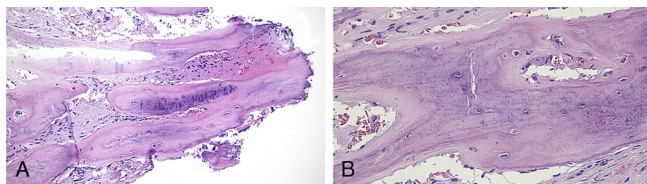


FIGURE 6. Biopsy of 4-week-old distraction site demonstrates evidence of new bone ossification in the bony regenerate.



FIGURE 7. Eighteen-month postsurgical follow-up for patient showing improved occlusion.

Analysis of their parents only revealed the presence of the duplication, although this was only phenotypically seen with the presence of hypodontia, suggesting a variable expression pattern. Most of the other cases are the result of cytogenetically detectable terminal duplications of 6p with variable breakpoints and often in the setting of an unbalanced translocation with concurrent partial monosomy for another chromosome. None of these cases were studied with molecular techniques, so it is difficult to know exactly which cases would include *RUNX2*. Villa et al¹⁵ reported that partial trisomy 6p is characterized by failure to thrive, recurrent respiratory infections, growth and psychomotor retardation, abnormal sutures, high forehead, choanal atresia, and other minor dysmorphic features. Detailed reports of cranial sutures are not available for these cases.

Our patient, who has 4 copies of *RUNX2*, exhibits a more severely affected phenotype along this continuum. To our knowledge, this quadruplication syndrome has never been reported. Additionally, his multisutural craniosynostosis is the most extensive associated with *RUNX2* we could find in reviewing the literature. This fact leads to the interesting possibility that this gene may regulate bone formation in a dose-dependent fashion. The more *RUNX2* transcription factor present, the more early and widespread abnormal ossification will occur. It is possible that other genes in the region may be responsible for this trend, but none so far have been shown to be clinically relevant. There is one example in the literature of high bone resorption in the face of overexpressed *RUNX2*, but it is in a mouse model.²¹ In contrast, our patient, although demonstrating midfacial retrusion and maxillary hypoplasia, actually possessed abnormal, osteopetrotic features to his facial bones, which resulted in a very challenging maxillary osteotomy and abnormally rapid bony callous ossification. Regardless, there is likely more complexity than a simple dose-dependent relationship, but thus far, it seems consistent in humans.

CCD and *RUNX2* duplication syndromes have such profound phenotypic differences because they share a common pathway, with *RUNX2* at the center. They exhibit a direct correlation between genotype and phenotype that is not often seen in the extreme complexity of the human body. Interestingly, *RUNX2* duplication syndromes share phenotypic similarities with other syndromic craniosynostoses, such as Crouzon syndrome. Presumably, these similarities stem from the common cellular pathways involved in the etiology of both. *FGFR2* mutations are thought to play a large role in the etiology of syndromic craniosynostosis. *FGF2* functions to phosphorylate and subsequently activate *RUNX2*. It is therefore likely that *RUNX2* is the common pathway shared by many of these syndromes, whether it is the primary cause or a downstream modulator.

Traditionally, *RUNX2* mutational analysis assessing for copy number has not been part of the routine genetic screening that accompanies the diagnosis of craniosynostosis. It is therefore likely that *RUNX2* duplications are more common than the literature would suggest. For a more accurate representation of the incidence of

RUNX2 duplications, this genetic locus should be evaluated on all patients with syndromic craniosynostosis, especially if routine screening is negative.

Our case has not only diagnostic but also therapeutic implications. Because of the theoretical promiscuity of osteoblastic differentiation that this mutation may bear on a molecular level, it was decided to abbreviate the latency period from the authors' usual protocol (48 hours instead of 3–5 days) to avoid premature consolidation of the Le Fort I osteotomy. Second, and interestingly enough, early ossification of the regenerate was evident at just 12 days into the consolidation phase both grossly and histologically. Traditionally, a consolidation period of 6 to 8 weeks is required [20]. This, however, is an area of much controversy. Review of the original literature on external halo distraction of the midface and the manufacturer's recommendations lead to the conclusion that the halo can be removed after 2 to 3 weeks of consolidation.²² Notably, these recommendations were derived from patients in the mixed dentition phase, who generally exhibit more rapid bone formation than an adult. Moreover, in patients who underwent transient maxillomandibular fixation in the rapid maxillary halo distraction protocol to refine the occlusion before rigid plate fixation, histological examination of the regenerate at the time of rigid fixation revealed only scattered fibroblasts in a collagen matrix, with absence of osteoblastic features.²⁰ This is in stark contrast to the histological findings of the regenerate in our patient (Fig. 6). The average time of distraction (mean, 15 days) and initial consolidation (2 weeks) in this maxillomandibular fixation subset of patients matches well with this current study. Extrapolating this observation, we propose that a tendency toward premature consolidation exists in the patient who has multiple copies of *RUNX2*, and this should impact our distraction protocols and postsurgical manipulation of the occlusion in these patients.²³ In vitro characterization of osteoblasts isolated from this patient is pending.

CONCLUSIONS

RUNX2 plays a central role in the regulation of osteoblast differentiation. Abnormal copy numbers of *RUNX2* lead to CCD, duplication syndromes, or even a Crouzon-like phenotype. The realization of its dose-dependent effect could prove to be invaluable. Our case report and other reports in the literature indicate that genetic associations of craniosynostosis are expanding and mandate thorough microarray testing of such individuals, especially if routine screening is negative. With more complete genetic screening in the future, we hope to identify more of these patients earlier in life. Surgical therapeutic implications of this case, such as the degree of difficulty in performing bony osteotomies, the implementation of shorter latency periods, and rapid distraction protocols, need to be heeded and further tested. Although surgical options may not change significantly, gene therapy will likely become an important alternative treatment modality for craniosynostosis as these signaling pathways are better elucidated.

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