CLINICAL REPORT

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A New Frontonasal Dysplasia Syndrome Associated With Deletion of the *SIX2* Gene

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The frontonasal dysplasias are a group of craniofacial phenotypes characterized by hypertelorism, nasal clefting, frontal bossing, and abnormal hairline. These conditions are caused by recessive mutations in members of the *aristaless* gene family, resulting in abnormal cranial neural crest migration and differentiation. We report a family with a dominantly inherited craniofacial phenotype comprised of frontal bossing with high hairline, ptosis, hypertelorism, broad nasal tip, large anterior fontanelle, cranial base anomalies, and sagittal synostosis. Chromosomal microarray identified a heterozygous 108.3 kilobase deletion of chromosome 2p21 segregating with phenotype and limited to the *sine oculis* homeobox gene *SIX2* and surrounding noncoding DNA. Similar to the human *SIX2* deletion phenotype, one mouse model of frontonasal dysplasia, *brachyrrhine*, exhibits dominant inheritance and impaired cranial base chondrogenesis associated with reduced *Six2* expression. We report the first human autosomal dominant frontonasal dysplasia syndrome associated with *SIX2* deletion and with phenotypic similarities to murine models of *Six2* Loss-of-function.

Key words: frontonasal dysplasia; Brachyrrhine; craniosynostosis; ptosis; hypertelorism

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INTRODUCTION

Frontonasal dysplasia (FND) syndromes include a wide phenotypic spectrum comprised of ocular hypertelorism, broad or cleft nose with abnormal nasal tip, frontal bossing, and hairline anomalies. FND type 1 involves the nose, philtrum, and eyelids (ptosis); type 2 includes alopecia, enlarged parietal foramina, and may be associated with genital abnormalities; type 3 has the most severe facial features and includes ocular abnormalities (microphthalmia or anophthalmia) and pinna malformations. Classical FND types 1–3 are caused by recessive mutations in the *Drosophila aristaless*-like homeobox genes (*ALX3*, *ALX4*, and *ALX1*, respectively) [Kayserili et al., 2009, 2012; Twigg et al., 2009; Uz et al., 2010; Kariminejad et al., 2014]. These transcription factors are expressed in neural-crest derived mesenchyme and lateral plate mesoderm and regulate cell fate determination and differentiation [ten Berge et al., 1998; Beverdam et al., 2001; Ettensohn et al., 2003; Kayserili et al., 2009, 2012; Kariminejad et al., 2014]. FND associated with craniosynostosis is seen in craniofrontonasal syndrome caused by mutation in *EFNB1* [Twigg et al., 2004; Wieland et al., 2004]. Functional alterations of these proteins result in abnormal development of midline craniofacial neural crest derivatives.

Here, we present the genetic analysis of a family with autosomal dominant FND segregating with chromosome 2p21 deletion encompassing the *SIX2* gene. This phenotype is consistent with a mouse model of *Six2* Loss-of function, *brachyrrhine*. [Lozanoff, 1993; Ma and Lozanoff, 1993, 1996; Lozanoff et al., 1994, 2001]. Together, these findings support that *SIX2* haploinsufficiency confers an autosomal dominantly transmitted FND in humans with phenotype similar to analogous craniofacial phenotypes of *Six2* and *brachyrrhine* (*Br*) mouse models.

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CLINICAL REPORT

The proband was referred to Genetics Clinic at 22 months of age for evaluation of craniosynostosis and dysmorphic features. She was the 2,756 g (10th centile) and 48.3 cm (25th centile) product of a full term vaginal delivery to a 37 year old gravida 2 para 2 woman after a pregnancy complicated by maternal hypothyroidism treated with levothyroxine. She was noted to have dysmorphic features including bilateral ptosis and epicanthus inversus at birth. Her primary care physician ordered a chromosome analysis which showed a normal female karyotype of 46,XX. Psychomotor development was normal and she reached normal milestones at the appropriate ages. Because she had a large anterior fontanelle, a 3D cranial computed tomography (CT) scan was performed documenting synostosis of the posterior sagittal suture. Physical examination at 22 months showed a healthy dysmorphic girl whose weight was 11.1 kg (35th centile), length 83 cm (35th centile), head circumference 50 cm (95th centile). The head was large with frontal bossing, large anterior fontanelle measuring 6.4 by 4.0 cm, flat nasal bridge, and wide nasal tip. She had ptosis and epicanthus inversus. She was hypeteloric with inner canthal distance 3.4 cm and outer canthal distance of 9.1 cm (both > 95th centile). There was bilateral ptosis with epicanthus inversus. The palate was intact but the lateral palatine ridges were wide. Dentition was normal. Ears were normally shaped but posteriorly rotated (see Fig. 1A,B). The remainder of the examination was normal, including the musculoskeletal and limb examination.

Her mother had a strikingly similar appearance to the proband, having a high broad forehead, flat nasal bridge, wide nasal tip, epicanthus inversus, and ptosis (Fig. 1C,D). Her mother's head circumference was 54.5 cm (50th centile) whereas her father's head circumference was 61 cm (>98th centile). Maternal grandparents and other relatives were not available for examination. Per maternal report, the proband's maternal uncle has very similar facial features, including hypertelorism, broad nasal bridge, and broad forehead. There was no known family history of hypertension or renal disease.

Review of the head CT with 3D reconstruction of the cranium confirmed large anterior fontanelle (Fig. 1E, arrow), persistent metopic suture (Fig. 1E, arrowhead), bilateral parietal foramina (Fig. 1F, arrowheads), and complete sagittal synostosis by 31 months (Fig. 1F, arrow). Subtle cranial base anomalies were present, including premature lateral spheno-occipital synchondrosis (Fig. 1H inset, arrowheads) and persistent craniopharyngeal canal (Fig. 1G, arrow, and Fig. 1H inset, arrow). Macrocephaly developed over that period to 53.3 cm (+2.8 SD). Renal ultrasound was normal, as were renal function testing for creatinine and electrolytes including calcium and phosphorous. Thyroid testing was normal. Her neurocognitive development has continued to be normal for age.

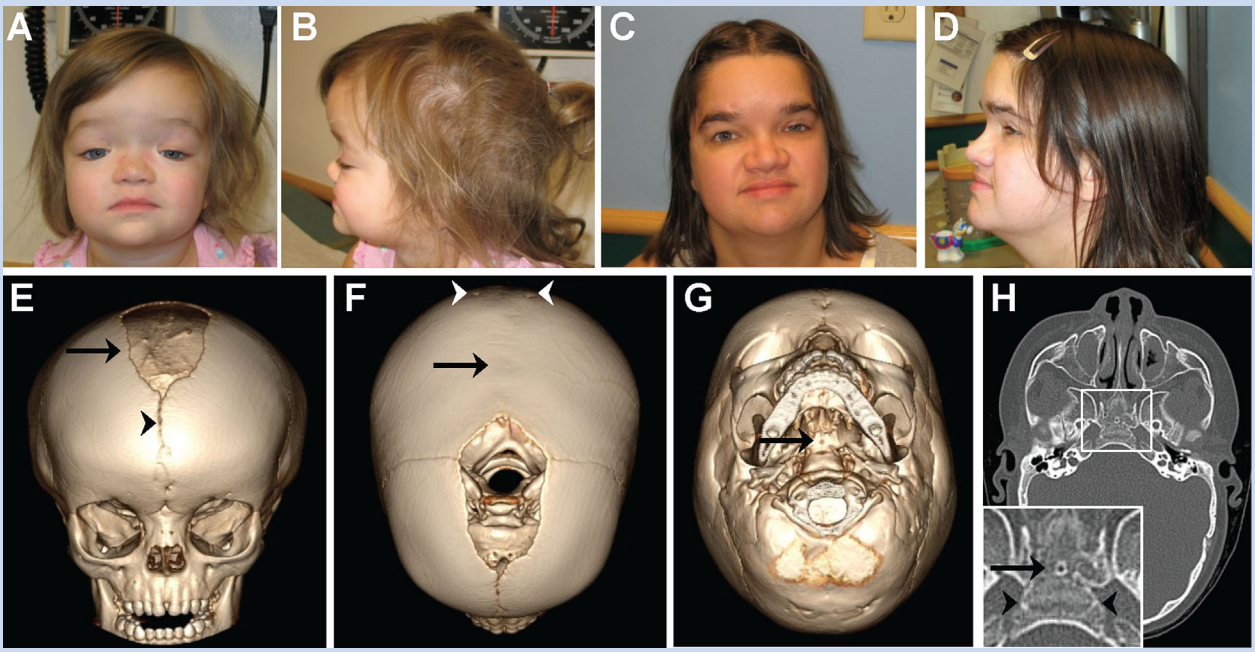

FIG. 1. Frontonasal dysplasia phenotype. A–B: Facial features of the proband at 21 months of age, including high hairline, frontal bossing, hypertelorism, ptosis, and broad nasal tip. C–D: Facial features of the proband's mother as an adult, including high frontal hairline, frontal bossing, hypertelorism, unilateral ptosis, broad nasal tip, and prognathia. E–H: Cranial CT 3D reconstruction for the proband at 31 months of age demonstrating wide anterior fontanelle (E, arrow), metopic synostosis (E, arrowhead), parietal foramina (F, arrowheads), sagittal synostosis (F, arrow), persistent craniopharyngeal canal (G and H, arrow), and premature lateral spheno-occipital synchondrosis (H, arrowheads).

Sequence analysis of genes associated with parietal foramina (*ALX4*, *MSX2*) and craniosynostosis (*FGFR1*, *FGFR2*, *FGFR3*, *TWIST)* did not reveal any pathogenic variants. Single nucleotide polymorphism (SNP)-based microarray (Human610-Quad BeadChip, Illumina) was performed and identified a 108.3 kilobase (kb) deletion of chromosome 2p21, with decreased log ratio and absence of heterozygosity across 39 markers (rs4953156 to rs17319875; chr2:45,189,763–45,298,085; GRCh37/hg19) (Fig. 2B). This included a complete and isolated deletion of the *SIX2* gene (chr2:45,232,324–45,236,542) and surrounding noncoding DNA (Fig. 2A). Parental testing confirmed that the *SIX2* deletion was present in the proband's mother and absent in her father. Sanger sequencing of *SIX2* exons in proband and mother revealed no pathogenic variants on the remaining allele. The *SIX3* gene

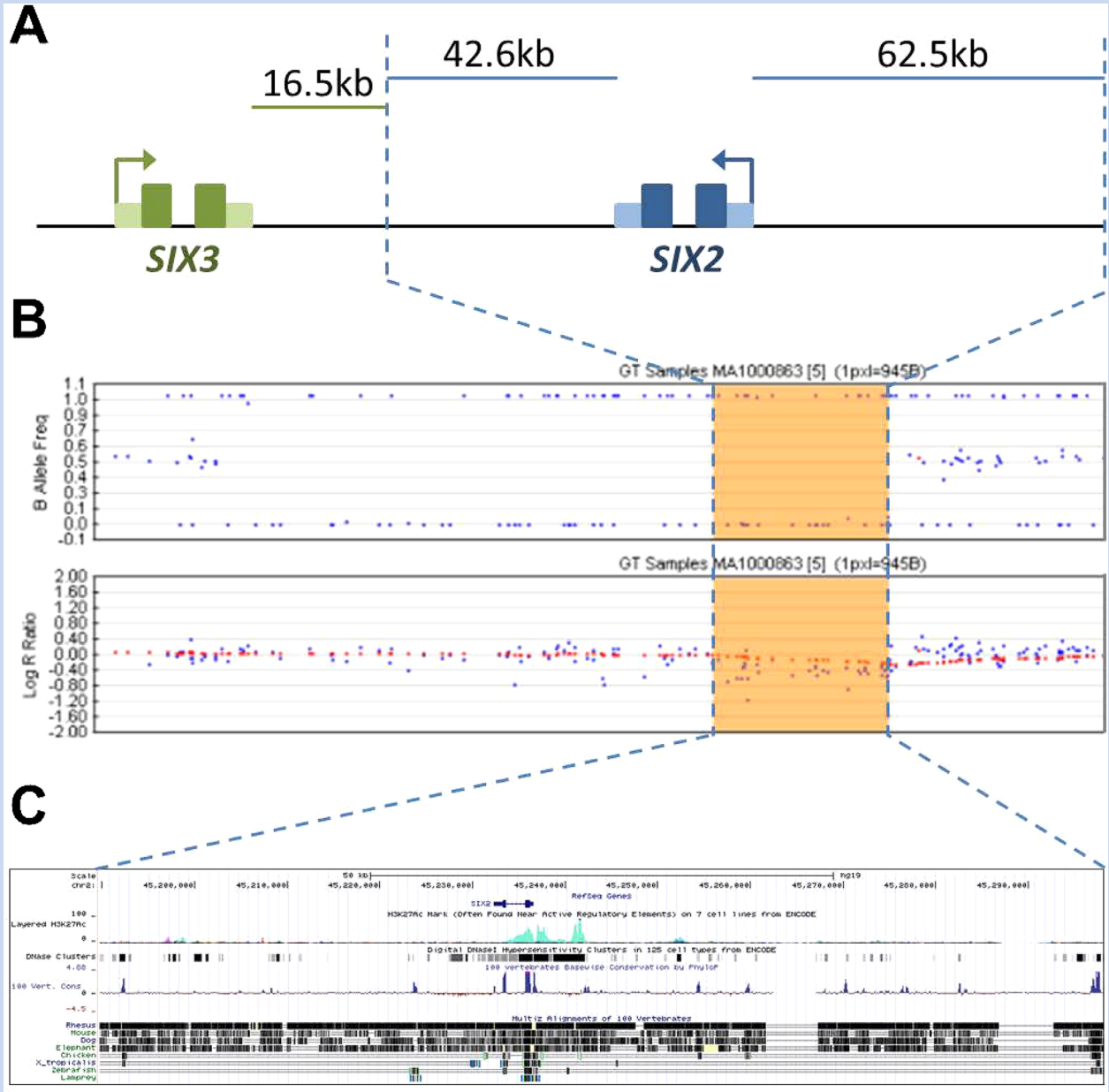

FIG. 2. 2p21 deletion including *SIX2*. A: Schematization of the 108.3 kb chromosome 2p21 deletion (blue dashed lines) encompassing the *SIX2* gene (blue) and surrounding noncoding DNA. The *SIX3* gene (green) is proximal to and not included in the deletion. B: Log ratio and B-allele frequencies depicted across the deletion. C: Regulatory elements within the deletion (chr2:45,189,763–45,298,085; hg19) depicted using UCSC genome browser (genome.ucsc.edu), including ENCODE regulatory elements predicted by H3K27Ac marks, DNAse hypersensitivity, and conservation in mammalian, vertebrate, and invertebrate species.

(chr2:45,169,037–45,173,216) proximal to the deletion was also sequenced in proband and mother, and no pathogenic variants were detected.

DISCUSSION

This case characterizes a novel dominantly inherited frontonasal dysplasia, similar to FND type 1 and associated with heterozygous deletion of the *SIX2* gene. The orthologues *Drosophila sine oculis* and murine *Six2* are critical for cell fate determination [Serikaku and O'Tousa, 1994]. In mouse, *Six2* is critical for cranial base formation, and loss-of-function models result in frontonasal dysplasia. Notably, FND types 1–3 are caused by recessive mutations in the *Drosophila aristaless* homeobox transcription factors (*ALX1*, *ALX3*, and *ALX4*) that are critical for differentiation of neural crest cells into midline midface craniofacial structures and the branchial arches [Mavrogiannis et al., 2001; Twigg et al., 2009; Uz et al., 2010]. Neither biochemical nor epistatic association of *aristaless*/*ALX* and *sine oculis*/*SIX* has been described. Together, this dominantly inherited FND phenotype implicates an additional family of homeobox transcription factors as candidate causes for FND.

Genes of the *SIX* family encode homeodomain transcription factors with high sequence similarity to the Drosophila gene *sine oculis* and are associated with branchial arch, ear, and renal malformations. Mutations in *SIX1* and *SIX5* cause branchio-oto-renal syndrome, characterized by cervical fistulae, ear anomalies, and renal hypodysplasia [Ruf et al., 2004; Hoskins et al., 2007]. Like other members of this family, the *SIX2* protein contains a DNA-binding homeodomain and an upstream SIX domain that functions in binding site specificity and protein-protein interactions [Boucher et al., 2000]. Missense dominant mutations in the human *SIX2* gene have been associated with isolated renal hypodysplasia without craniofacial involvement [Weber et al., 2008]. However, these demonstrate incomplete penetrance or multifactorial etiology, as parents of multiple patients carrying these mutations have normal kidney function. In our case, neither the proband nor her mother has a history of hypertension or other signs of renal dysfunction or dysplasia. One possibility is that whole gene deletion results in haploinsufficiency, whereas missense mutations produce an abnormal protein with altered function, either gain- or partial loss-of-function.

Targeted deletion of the murine *Six2* gene results in craniofacial defects, renal hypodysplasia, and neonatal lethality in homozygous animals [Self et al., 2006]. *Six2* mutant mice have cranial base deficits resulting in a compressed cranial appearance, with no cartilage between the basioccipital, basisphenoid, and presphenoid bones [He et al., 2010]. Additionally, there is absent intrasphenoidal and reduced spheno-occipital synchondrosis. This reduction of cartilage and impaired synchondrosis is likely due to premature chondrocyte maturation and decreased proliferation, thereby prematurely depleting the pool of cartilagenous progenitor cells [He et al., 2010]. No craniofacial or renal phenotype has been reported for heterozygous *Six2* mutant mice. On the other hand, the radiation-induced mouse mutant *brachyrrhine (Br)* carries a semi-dominant mutation resulting in frontonasal dysplasia (FND) and renal hypoplasia [McBratney et al., 2003; Fogelgren et al., 2008]. The heterozygous mutant (*Br*/+) survives to adulthood, while homozygous mutants (*Br*/*Br*) die at birth. Homozygous *brachyrrhine* mice (*Br*/*Br*) have severe frontonasal dysplasia, anterior cranial base malformations including unfused trabecular cartilage and absent presphenoid bone, midfacial retrusion with resulting retrognathia, and renal hypoplasia resulting in intrauterine growth restriction and embryonic lethality [Lozanoff, 1993; Ma and Lozanoff, 1993, 1996; Lozanoff et al., 1994, 2001; Fogelgren et al., 2009]. Similar to *Six2* mutant mice, chondrogenesis is impaired.

The *brachyrrhine* heterozygous mouse (*Br*/+) has an intermediate phenotype including midface hypoplasia, hypertelorism, and sphenoidal abnormalities. Frontonasal dysplasia and anterior cranial base malformations in heterozygous mice (*Br*/+) include fused trabecular cartilage lacking lateral chondrification and absent orbital cartilages. The *Br*/+ craniofacial phenotype is most similar to the human *SIX2* heterozygous deletion phenotype in terms of viability, frontonasal dysplasia, and cranial base abnormalities, particularly of the sphenoid and spheno-occipital synchondrosis. Linkage and expression analyzes link the *brachyrrhine* mouse phenotype to a critical region containing the *Six2* gene and highly overlapping the homologous human 2p21 deletion. Notably, in the *brachyrrhine* mouse no mutation was found in the *Six2* coding sequence or 1.5 kb upstream of the promoter region. However, the *brachyrrhine* mouse has a dose dependent decrease in *Six2* expression, suggesting existence of a novel distal cis-regulatory element [Fogelgren et al., 2008]. Therefore, deletion of a cis-regulatory element of *SIX2* may contribute to the frontonasal dysplasia observed in our patients (Fig. 2C).

Molecular mechanisms governing *SIX2* expression and function appear to play orthologous roles in human and mouse craniofacial development. *Six2* expression in the craniofacial mesenchyme is regulated by *Hox* family member *Hoxa2*, which regulates the development of second branchial arch derivatives that comprise the stapes, styloid, and lesser hyoid through inhibition of *Six2* expression [Kutejova et al., 2005]. *Hoxa2* overexpression in chicken or mouse results in abnormalities of the facial skeleton and decreased levels of *Six2* in the cranial mesenchyme [Kanzler et al., 1998; Creuzet et al., 2002]. Other studies indicate that, in the second branchial arch, *Six2* regulates *Igf1* and *Igfbp5* in bone development, and dysregulation of this pathway results in bone overgrowth in *Hoxa2* mutants [Kutejova et al., 2005, 2008]. Noncoding *Six2* regulatory elements (Fig. 2C) are likely pleiotropic, as *Six2* has shown to be repressed by *Hoxa2* and activated by *Hox11* at a single enhancer [Yallowitz et al., 2009].

In conclusion, *SIX2* haploinsufficiency results in frontonasal dysplasia likely due to altered neural crest cell fate specification and differentiation in the branchial arches. Contributions of other genes regulating *SIX2* expression, including *HOX* genes, and of factors downstream of *SIX2* function, including *IGF* genes, likely constitute a significant pathway in craniofacial development. This pathway may intersect with the functions of the *ALX* gene family. Therefore, *SIX2* and other genes within this pathway should be considered in patients exhibiting similar frontonasal dysplasia phenotypes.

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