A Novel \textit{SIX3} Mutation Segregates With Holoprosencephaly in a Large Family

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Abstract

Holoprosencephaly is the most common structural malformation of the forebrain in humans and has a complex etiology including chromosomal aberrations, single gene mutations and environmental components. Here we present the pertinent clinical findings among members of an unusually large kindred ascertained over 15 years ago following the evaluation and subsequent genetic work-up of a female infant with congenital anomalies. A genome-wide scan and linkage analysis showed only suggestive evidence of linkage to markers on chromosome 2 among the most likely of several pedigree interpretations. We now report that a novel missense mutation in the \textit{SIX3} holoprosencephaly gene is the likely cause in this family. Molecular genetic analysis and/or clinical characterization now show that at least 15 members of this family are presumed \textit{SIX3} mutation gene carriers, with clinical manifestations ranging from phenotypically normal adults (non-penetrance) to alobar holoprosencephaly incompatible with postnatal life. This particular family represents a seminal example of the variable manifestations of gene mutations in holoprosencephaly and difficulties encountered in their elucidation.

Keywords

holoprosencephaly; HPE; \textit{SIX3}

INTRODUCTION

Holoprosencephaly (HPE) occurs due to incomplete midline cleavage of the developing brain during the third and fourth weeks of gestation and is the most common structural malformation involving the human forebrain. HPE affects about 1 in 250 gestations, but this falls to only about 1 in 10,000 live births [Matsunaga and Shiota, 1977; Leoncini et al., 2008]. Three classical recognizable degrees of severity, defined by the extent of brain malformation, have been described: alobar, semilobar, and (frontal) lobar HPE [Muenke and Beachy, 2000; Cohen, 2006]. In addition, a posterior form of lobar HPE designated middle interhemispheric variant (MIHV or “syntelencephaly”) has also been recognized [Barkovich and Quint, 1993]. Clinical

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manifestations typically include characteristic craniofacial anomalies, developmental disabilities, pituitary insufficiency, and seizures. Craniofacial findings tend to correlate with the severity of brain anomalies. Individuals at the severe end of the spectrum may manifest severe microcephaly, a single eye (cyclopia), anophthalmia or microphthalmia, a mound of tissue located just above a fused eye or two very closely spaced eyes designated a proboscis, absent nasal septum, and midline or bilateral cleft lip and palate. At the less severe end of the spectrum, sometimes termed an HPE “microform,” patients may have microcephaly, hypotelorism, and single maxillary central incisor without the structural brain changes of HPE. Severely affected patients do not typically survive the neonatal period. Less severely affected individuals may have a normal lifespan [Cohen, 2006; Dubourg et al., 2007].

HPE can be caused by single gene mutations, although mutations in the four most common HPE-associated genes account for less than 25% of patients with HPE [Lazaro et al., 2004; Muenke, unpublished data]. Sonic hedgehog (SHH) was the first causal gene discovered and is still the only HPE locus identified by linkage analysis [Muenke et al., 1994; Roessler et al., 1996]. Since this promising beginning, linkage analysis has been less successful as a gene discovery method, and further elucidation of HPE-associated genes has largely proceeded through testing for mutations in genes in known SHH-related pathways and/or in regions identified in individuals with HPE and chromosomal anomalies [Brown et al., 1998; Gripp et al., 2000; Ming et al., 2002]. For example, GLI2 was known to be one member of a family of transcriptional mediators for SHH, and examination of human pedigrees revealed HPE-segregating mutations in GLI2. Families segregating mutations of GLI2 or other HPE causal genes highlight the markedly variable expressivity observed in patients with HPE-segregating mutations, suggesting additional environmental or genetic influences acting as modifying factors [Roessler et al., 2003]. In other words, a mutation in a gene such as GLI2 is necessary but not sufficient for HPE, and other genetic or environmental factors must be present for complete phenotypic manifestation [Ming and Muenke, 2002].

SIX3 was identified as an HPE candidate gene in individuals with HPE and structural rearrangements of chromosome 2p21 [Schell et al., 1996; Wallis et al., 1999]. Heterozygous mutations in SIX3 are now known in over 120 individuals representing over 60 different kindreds with HPE, with clinical findings again demonstrating incomplete penetrance and highly variable expressivity [Wallis et al., 1999; Nanni et al., 2000; Pasquier et al., 2000, 2005; Dubourg et al., 2004; Lazaro et al., 2004; Ribeiro et al., 2006; Bendavid et al., 2006a,b; El-Jaick et al., 2007; Domené et al., 2008; Muenke, unpublished data].

The vertebrate Six genes encode a family of transcription factors orthologous to the Drosophila sine oculis (“without eyes”) gene that functions in the developing fly’s visual system. Vertebrate Six3 is involved in midline forebrain and eye formation in several organisms [Oliver et al., 1995; Kobayashi et al., 1998; Gestri et al., 2005]. Known biological properties of vertebrate Six3 include transcriptional repression of BMP, Wnt and Nodal targets through complex(s) formed with Groucho, influence over cellular fate in the developing forebrain through interactions with Geminin, activation of lens specification during eye formation, and, importantly, regulation of SHH in the ventral forebrain [Kobayashi et al., 2001; Lagutin et al., 2003; Del Bene et al., 2004; Liu et al., 2006; Inbal et al., 2007; Geng et al., 2008]. Proteins encoded by the Six genes typically contain a DNA-binding homeobox domain and an upstream SIX domain that can recruit factors to accomplish transcriptional activation or repression [Kawakami et al., 1996; Granadino et al., 1999].

Here we present a large kindred initially ascertained due to birth of an infant with alobar HPE. Subsequent study of her family showed that at least 15 members in 5 generations segregate a mutation in SIX3.
MATERIALS AND METHODS

Blood samples were sent to the Muenke laboratory for analysis after obtaining appropriate consent. Mutation detection was performed by PCR amplification of all exons, single-strand conformational polymorphism (SSCP), and denaturing high performance liquid chromatography (dHPLC) screening, and bidirectional sequencing as described previously [Wallis et al., 1999; Domené et al., 2008]. Direct sequencing of the proposita’s SHH gene was performed, by techniques previously described [Roessler et al., 1996]. After mutation identification, a retrospective genome-wide scan was performed using microsatellite markers genotyped by DeCODE genetics (1.5 cM average spacing, 2000 markers) under contract agreement. Mendelian errors were detected using PEDCHECK and were removed from analysis [O’Connell and Weeks, 1998]. Parametric and non-parametric linkage analyses were performed using GENEHUNTER-PLUS [Kruglyak et al., 1996; Kong and Cox, 1997]. Given the evidence of reduced penetrance and potential misclassification of phenocopies as HPE microforms, parametric linkage analysis was run with autosomal dominant penetrance parameters of 0.10 for genotype aa and 0.80 for genotypes Aa or AA; an allele frequency of the disease causing mutation was set at 0.001 for all parametric analyses. For suggestive regions of linkage, haplotyping was performed using MERLIN’s best function [Abecasis et al., 2001]. Haplotypes were evaluated using HAPLOPAINTER to define minimal critical genomic intervals by examining recombination events [Thiele and Nürnberg, 2005].

RESULTS

Family Description

The family (Fig. 1 and Fig 2) was ascertained at Indiana University Hospital over 15 years ago (by C.M. and W.B.D.) following evaluation of a 1-day-old girl (IV.19) born at term with congenital anomalies including macrocephaly, severe hypotelorism, a short nose with upturned nares, a hypoplastic philtrum consisting of a thin vertical groove, low-set ears, a slightly small jaw, and normal palate. Her fingers were overlapping (finger 3 overlapping 4 bilaterally) with slight contractures and deep but otherwise normal palmar creases. Cranial CT revealed alobar HPE and hydrocephalus (Fig. 2). Interestingly, DNA from this child and her family comprised one of the first complete family sets sent to the Muenke lab in 1990 for research testing. This sample was eventually screened for mutations in SHH, ZIC2, SIX3, and TGIF by SSCP analysis. We now reflect that the apparent lack of band-shift with SIX3 specific primers represents our first obvious instance of false negative screening results [Muenke, unpublished data].

Subsequently, over 20 members of the patient’s extended family were examined by the authors, largely by W.B.D. (Fig. 1 and Table I for details). Findings consistent with full HPE occurred in three individuals including the proposita. Six other children died in early infancy of unknown causes. At least nine individuals had a subtle facial microform consisting of a sharp angular nose with either frank hypotelorism or a mildly narrow nasal bridge (Fig. 2). One young man (IV.4) had bilateral microphthalmia, microcornea, and coloboma of the iris, choroid and retina with reportedly normal intelligence. We were unable to perform an examination or review photographs on this individual. One girl (IV.20) had isolated mental retardation (her IQ was about 50), microcephaly, a normal facial appearance, and a normal brain MRI. We thought that she had an HPE microform for many years, but she was later found not to carry the SIX3 mutation.

Genetic Testing

The original samples were ascertained over 15 years ago, prior to identification of HPE-causing genes. The initial linkage analysis was done using data from 21 individuals (including
individual IV.20) and modeled for reduced penetrance and phenocopies, but the results were inconclusive. DNA samples from this family were later tested by SSCP as candidate HPE genes were identified through other studies. Though the proposita’s sample did not initially reveal positive findings, dHPLC on her maternal uncle (III.5) ultimately showed a positive finding in SIX3. Direct sequencing detected a missense mutation in the SIX domain of SIX3: c.339G>T, resulting in p.W113C. This mutation results in an almost complete loss of function, as demonstrated by functional studies using a zebrafish model [Domené et al., 2008]. Sequencing of SIX3 was performed on 8 individuals, 6 of whom were found to have the mutation: the proposita (IV.19), her mother (III.7), two maternal aunts (III.1, III.8), a maternal uncle (III.5), and her maternal grandmother (II.2). Two individuals tested negative for SIX3 mutations: a maternal uncle (III.11) and his daughter, a maternal first cousin (IV.20). Finally, because of recent work showing direct Six3-Shh interactions [Geng et al., 2008], SHH sequencing was performed in the proposita and did not show any sequence variations.

When a retrospective linkage analysis was performed with the information garnered through direct sequencing, three signals overlapped or neighbored previously described genes for HPE: SIX3 on chromosome 2p (Lod = 1.12, NPL = 2.57), PTCH1 on chromosome 9q (NPL = 2.36) and TGIF on chromosome 18p (NPL = 2.57). However, none of these signals reached the level of statistical significance. Haplotype analysis provided further evidence against both PTCH1, as one affected individual did not share the linked haplotype, and TGIF due to a recombination event 300 Kb upstream of the gene.

Haplotype analysis in the SIX3 region using available DNA samples demonstrated that 9 individuals had a shared haplotype, and one individual in generation I must have carried the mutation as multiple offspring were affected. Two key crossover events in individuals III.5 (centromeric) and III.8 (telomeric) identified a SIX3 mutation-containing haplotype delimited by markers D2S2328 and D2S2294. This analysis identified 3 individuals in addition to those found by direct sequencing who co-segregated the haplotype containing the mutated SIX3 gene. Direct mutation testing was not performed on these individuals, as samples were no longer available. One of them, a maternal first cousin (IV.14), had a normal phenotype, while two, a maternal half-sister (IV.15) and maternal great uncle (II.5), had an HPE microform consisting of a sharply angular nose without apparent hypotelorism.

**DISCUSSION**

Here we report the largest single kindred with the most confirmed cases of a molecularly-defined SIX3 mutation. There is evidence that nine individuals in four generations carry the mutation, as shown by either direct sequencing or the presence of the SIX3 mutation-specific haplotype. At least an additional 4 individuals had clinical signs of HPE spectrum, and 2 individuals were presumed carriers, yielding presumptive positive findings in 15 individuals among 5 generations. It is possible that fewer individuals had the mutation. For example, individual II.5 might carry the same haplotype as those with the mutation, but not the mutation itself, and individual III.27 could have HPE due to a reason other than the mutation in SIX3. While a common mutation in at least these 15 individuals is the simplest explanation, it cannot be assumed that Occam’s razor provides the only explanation.

Study of this kindred recalls lessons learned in previous studies of HPE and other complex traits. First, this pedigree demonstrates the difficulties in the use of linkage analysis as a tool to discover disease-causing genes. Phenocopies are one complicating factor. Here, initial linkage analysis including the apparent phenocopy (IV.20) and her parent (III.11) did not demonstrate any meaningful linkage to the SIX3 locus. Even when direct mutation testing demonstrated that the phenocopy did not possess the mutation, repeat linkage analysis did not achieve statistical significance at the SIX3 locus.

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Second, this pedigree demonstrates well the incomplete penetrance and variable expressivity seen in other kindreds with HPE. As described according to the “multiple hit” theory [Ming and Muenke, 2002] other genetic and/or environmental factors likely affect phenotypic severity. In fact, the phenocopy described here (IV.20) may have been affected by these “modifying” factors, but may not herself have possessed the mutation in SIX3. Despite the recent elucidation of the potential for direct Six3-Shh genetic interactions in animals, there are no known cases of HPE in humans due to simultaneous mutations in SIX3 and SHH. Perhaps exonic coding mutations in SHH are not found simultaneously with similar changes in SIX3, but SHH regulatory changes not routinely tested in CLIA laboratories may co-occur.

Third, this kindred illustrates the challenges of genetic counseling in cases such as these. Despite the fact that this sequence change was shown to be a significant loss-of-function mutation, the presence of the mutation in SIX3 was seen in both unaffected individuals and in individuals with severe HPE. In these circumstances, genetics professionals must be aware of this range of possibilities, and should attempt to incorporate this understanding both in the interpretation of test results and in making prognoses.

Finally, clinical examination of individuals with the SIX3 mutation who were considered to possess the HPE microform often had a sharply angular nose (described as “knifelike” by one clinician) without true hypotelorism or microcephaly. The correlation of this phenotype with the presence of the mutation was more obvious in retrospect, and highlights the fact that genetic disorders may manifest in ways not exactly as traditionally described. Along these lines, 15 years passed between the initial genetic consultation on the proposita and the elucidation of the molecular cause. This extended diagnostic period demonstrates the importance of perseverance despite initially negative studies, including applying new technology and testing newly discovered genes.

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Figure 1.
Pedigree of family.
Figure 2.
a: Proposita (IV.19) with macrocephaly, hypotelorism, hypoplastic philtrum, and low-set ears; (b,c) head CT showing alobar HPE and hydrocephalus; (d) mother (III.7); (e) aunt (III.8); (f) grandmother (II.2); (g) great-uncle (II.5). All individuals had evidence for the presence of the \textit{SIX3} mutation. The proposita’s relatives show various signs of microform HPE, with varying degrees of microcephaly, hypotelorism, and thin nasal bridges.
**TABLE I**

Phenotypic Data of Members of Family

| Individual | Age at exam (years) | OFC (%) | ICD (%) | OCD (%) | Death in infancy | HPE | MR | MC (−2 SD) | HoT | SAN | HP | MO |
|------------|---------------------|---------|---------|---------|-----------------|-----|----|------------|-----|-----|----|----|
| II.2       | 70                  | 10–25   | 3–25    | 3–25    | −               | −   | −  | −          | +   | +   | −  | −  |
| II.5       | 68                  | 10      | 50–75   | 50–75   | −               | −   | −  | −          | +   | +   | −  | −  |
| II.12      | 53                  | 3–10    | 50      | 25–50   | −               | −   | −  | −          | −   | −   | −  | −  |
| III.1      | A                   | 50–75   | 75–97   | 75–97   | −               | −   | −  | −          | −   | −   | −  | −  |
| III.2      | 50                  | 75–97   | 75–97   | 75–97   | −               | −   | −  | −          | −   | −   | −  | −  |
| III.5      | A                   | 25      | 3–25    | 25–50   | −               | −   | −  | −          | +   | −   | −  | −  |
| III.7      | 39                  | <3      | <3      | <3      | −               | −   | −  | +          | +   | −   | −  | −  |
| III.8      | 37                  | <3      | 25–50   | 3–25    | −               | −   | −  | +          | +   | −   | −  | −  |
| III.11     | 31                  | 50      | 50–75   | 50–75   | −               | −   | −  | −          | −   | −   | −  | −  |
| III.12     | A                   | …      | …      | …      | −               | −   | −  | −          | +   | −   | −  | −  |
| III.27     | …                   | …      | …      | …      | +               | +   | …  | …          | …   | …   | …  | …  |
| IV.3       | …                   | …      | …      | …      | +               | …   | …  | …          | …   | …   | …  | …  |
| IV.4       | …                   | …      | …      | …      | −               | −   | −  | −          | +   | +   | +  | +  |
| IV.5       | 27                  | 50–75   | 75      | 50–75   | −               | −   | −  | −          | −   | −   | −  | −  |
| IV.6       | 24                  | 10      | 3–25    | 3–25    | −               | −   | −  | −          | −   | −   | −  | −  |
| IV.11      | 22                  | 25      | 50      | 25–50   | −               | −   | −  | −          | −   | −   | −  | −  |
| IV.12      | 21                  | 10–25   | 50–75   | 50–75   | −               | −   | −  | −          | −   | −   | −  | −  |
| IV.13      | …                   | …      | …      | …      | +               | +   | …  | …          | …   | …   | …  | …  |
| IV.14      | 9                   | >97     | 25–50   | 25–50   | −               | −   | −  | −          | −   | −   | −  | −  |
| IV.15      | 28                  | 10      | 3       | 25–50   | −               | −   | −  | −          | +   | +   | −  | −  |
| IV.16      | 16                  | 10–25   | 25–50   | 50–75   | −               | −   | −  | −          | −   | −   | −  | −  |
| IV.17      | 13                  | 25      | 3–25    | 3–25    | −               | −   | −  | −          | +   | +   | −  | −  |
| IV.18      | 5                   | 25–50   | 25–50   | 25–50   | −               | −   | −  | −          | +   | +   | +  | −  |
| IV.19      | 1 day               | >97     | <3      | <3      | +               | +   | …  | −          | +   | +   | +  | −  |
| IV.20      | 4                   | <3      | 3–25    | 3–25    | −               | −   | +  | +          | −   | −   | −  | −  |

+ present; (+) likely present; (−) absent; (…) no data or not examined.

OFC, occipitofrontal head circumference; ICD, inner canthal distance; OCD, outer canthal distance; HPE, holoprosencephaly; MR, mental retardation; MC, microcephaly; SD, standard deviations; HoT, hypotelorism; HP, hypoplastic philtrum; MO, microophthalmia; A, adult.