Mutations in ZIC2 in human holoprosencephaly: description of a novel ZIC2 specific phenotype and comprehensive analysis of 157 individuals.

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Mutations in *ZIC2* in Human Holoprosencephaly: Comprehensive Analysis of 153 Individuals and Description of a Novel *ZIC2*-Specfic Phenotype

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Abstract

Holoprosencephaly (HPE) is the most common malformation of the human forebrain, and may be due to cytogenetic anomalies, teratogens, occur in the context of a syndrome, or be due to mutations in single genes associated with non-syndromic HPE. Mutations in *ZIC2*, a transcription factor located on chromosome 13q32, are the second-most common cause of non-syndromic, non-chromosomal HPE. Blood samples from over 1000 individuals with HPE-spectrum disorders and their relatives were analyzed for sequence variations in *ZIC2*. We examined clinical details and included all other known previously published and unpublished cases of mutations in *ZIC2* through a literature search and collaboration with other centers. We find mutations in *ZIC2* in 8% of probands with HPE, and describe 153 individuals from 116 unrelated kindreds, including 137 patients with molecularly-determined mutations in *ZIC2* and 16 patients with deletions of the *ZIC2* locus. Unlike HPE due to mutations in other genes, the vast majority of cases are sporadic and the proportional distribution of HPE types differs significantly from previously published analyses of non-chromosomal non-syndromic HPE. Furthermore, we describe a novel facial phenotype in patients with mutations in *ZIC2* which includes bitemporal narrowing, upsplanting palpebral fissures, a short nose with anteverted nares, and a broad and well-demarcated philtrum, and large ears. This phenotype is distinct from the standard facial dysmorphisms associated with non-chromosomal, non-syndromic HPE. Our findings show that HPE due to mutations in *ZIC2* is distinct from that due to mutations in other genes. This may shed light on the

mechanisms that contribute to the formation of the face and the forebrain and may help direct genetic counseling and diagnostic strategies.

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Holoprosencephaly (HPE) is the most common malformation of the human forebrain, and results from failed or incomplete forebrain cleavage early in gestation. HPE occurs in 1 in 250 gestations, though the vast majority of conceptions with HPE do not survive to birth[1,2]. HPE is categorized by the degree of forebrain separation into alobar, in which there is no interhemispheric division, semilobar, and lobar HPE, from the most to least severe type. More recently, middle interhemispheric variant (MIHV) HPE has also been described, which includes failed separation of the posterior frontal and parietal lobes[3-6]. The distribution of HPE types in both living patients and deceased fetuses with non-chromosomal, non-syndromic HPE has been estimated to be 22% alobar, 45% semilobar, and 33% lobar HPE[7].

Common clinical features among patients with HPE include neurological impairment (often severe), seizures, diabetes insipidus, and characteristic dysmorphic facies. Traditionally, it is thought that in HPE "the face predicts the brain": in other words, more severe craniofacial anomalies correlate with more severe neuroanatomic findings.[4] At the most severe end of the spectrum, facial features in patients with alobar HPE may include cyclopia and a proboscis (a tubular nasal structure located above the fused eyes). Other, more common facial dysmorphisms in less-severely affected patients include microcephaly (though hydrocephalus can lead to macrocephaly), hypotelorism, a flat nasal bridge, and cleft lip and/or palate. At the least severe end of the spectrum, termed microform HPE, patients may have subtle features such as mild microcephaly,

hypotelorism, single maxillary central incisors (SMCI) without appreciable CNS anomalies on conventional neuroimaging. These individuals are often identified due to the presence of a severely affected relative[6,8-9].

HPE is etiologically heterogeneous, and may be caused by cytogenetic anomalies, teratogenic influences, occur in the context of a syndrome, or be due to mutations in one of over 10 HPE-associated genes[6-8,10-12]. In patients with HPE who have a normal chromosome analysis, a typical initial diagnostic strategy is to screen for mutations in four genes: *SHH* [MIM 600725], *ZIC2* [MIM 603073], *SIX3* [MIM 603714], and *TGIF* [MIM 602630]. Mutations in these genes can arise *de novo* or may be found in multiple members of large families segregating HPE-spectrum anomalies. In large kindreds, family studies demonstrate the incomplete penetrance and highly variable expressivity of these mutations[3-4,6,13].

ZIC2, located at chromosome 13q32, was first identified as an HPE candidate gene due to individuals with brain anomalies who were found to have deletions involving the long arm of chromosome 13. Subsequent analysis of patients with HPE identified mutations in *ZIC2*[14-16]. Mutations in this gene have previously been thought to be the second-most-common identified cause of non-chromosomal non-syndromic HPE (after mutations in *SHH*). In recent estimates, at least 3% of probands with HPE have mutations in *ZIC2*[6,17]. *ZIC2* codes for a transcription factor which plays several roles in neurological development. Early in development, ZIC2 is predicted to play a role in axial midline establishment; later, ZIC2 appears to affect the development of the dorsal telencephalon[18,19]. This latter role may explain the occurrence of neural tube defects in individuals with mutations in *ZIC2*, as well as the presence of MIHV-type HPE, though

this type can be seen in HPE due to mutations in other genes as well[20]. Mouse models show that complete absence of Zic2 activity results in HPE due to mid-gastrulation failure of axial midline development, homozygous hypomorphic alleles result in normal gastrulation but dorsal forebrain malformations at later stages, and heterozygotes for null alleles are phenotypically normal. However, features in homozygous null mice may recapitulate the entire spectrum of HPE severity, suggesting that the phenotypic consequences of mutations depend on the perturbed developmental stage and may be affected by interacting genes[17,19,21-22]. Of note, it has been suggested that mutations in *ZIC2* may result in HPE, but often do not result in facial features typically seen in human patients with HPE due to mutations in other genes[23].

Here we present clinical and genetic data on all known individuals with mutations in *ZIC2*, over half of whom were identified through our laboratory via direct sequencing. We also present data on individuals with deletions of the *ZIC2* locus ascertained by Multiplex Ligation-dependent Probe Amplification (MLPA) and Fluorescence in Situ Hybridization (FISH), chromosome analysis, or by oligonucleotide array comparative genomic hybridization. Through this comprehensive evaluation, we can identify specific characteristics of these individuals that can differentiate the phenotypic findings in patients with HPE due to *ZIC2* mutations from patients with HPE due to other genetic causes.

Blood samples from approximately 800 individuals with HPE-spectrum disorders and their relatives were collected prospectively over 18 years. These samples were analyzed for potential sequence variations in the *ZIC2* gene under our NHGRI-approved

brain research protocol after appropriate consent had been obtained. A strategy for screening the *ZIC2* gene has previously been described[17]. Clinical history, photographs, and neuroimaging were reviewed where available, again after appropriate consent was obtained. Three patients were seen at the National Institutes of Health for a comprehensive evaluation. Collaborators sent us de-identified clinical and laboratory data on patients with identified mutations in *ZIC2*.

A Medline search was conducted to find previously reported cases of holoprosencephaly due to mutations in *ZIC2*. The key words and patient terms included "*ZIC2*", "holoprosencephaly", "HPE", "13q", and "13q32". References were also obtained from papers found through the literature search. As loci nearby *ZIC2* may contribute to brain malformations and there have been numerous reported cases of deletions of 13q with unreported clinical and genetic characterizations, only cases which had clear HPE and definitive deletion of the *ZIC2* locus without involvement of other chromosomes were considered. Cases were used from the following papers and abstracts: [Brown et al., 1993]; [Brown et al., 1995]; [Brown et al., 1998]; [Chen et al., 1998]; [Nanni et al., 2000]; [Gutierrez et al., 2001]; [Orioli et al., 2001]; [Brown et al., 2001]; [Marcorelles et al., 2002]; [Dubourg et al., 2004]; [Brown et al., 2005]; [Júnior et al., 2006]; [A. Paulussen et al., 2008, Eur. Soc. of Hum. Genet., abstract.]; [Roessler et al., 2009]; [Quélin et al., 2009][14-16,23-32].

We describe a total of 153 patients, including 137 patients from 100 unrelated kindreds with molecularly-determined mutations in *ZIC2*, 7 patients with deletions of *ZIC2* ascertained by FISH testing and 9 patients with deletions of *ZIC2* ascertained by

chromosome analysis or by oligonucleotide microarray. By direct sequencing of DNA samples of an unselected group of unrelated patients with HPE, 8.25% (99/1200) have mutations in *ZIC2* (NIH: 49/285; Rennes: 41/532; Maastricht: 9/86). Additional cases among the approximately 800 tested in our laboratory were ascertained through screening methodology, including screening methods involving single-strand conformational polymorphism (SSCP) analysis and denaturing high-performance liquid chromatography (dHPLC).

Of note, in the descriptions below, unless otherwise stated, results refer only to individuals with molecularly determined mutations in *ZIC2*. Denominators differ among findings, as the prevalence of each phenotypic manifestation was calculated only where data was available for that specific finding. A summary of all patients is presented in the Supplementary Table.

Inheritance

Among probands in whom parents were available for testing, mutations were found to be *de novo* in 74% (49/66), maternally inherited in 18% (12/66), and paternally-inherited in 8% (5/66) of patients. There were no kindreds in which mutations or affected individuals were identified in more than 2 generations. However, in 4 cases, pedigree analysis showed that a mutation appeared to be inherited from a parent who had multiple affected children but for whom mutation testing was negative, implying either allele drop-out or, more likely, germline mosaicism.

HPE type

Prevalences of HPE types for both all described individuals and probands are presented as tables 1 and 2. Among patients with HPE, the distribution of classic HPE types (not

including MIHV-type HPE) among patients with mutations in *ZIC2* differs significantly from a previously published analysis of HPE distribution among patients with non-chromosomal, non-syndromic HPE ($\chi^2 = 16.401$; $p = 0.0003$)[7]. Patients with mutations in *ZIC2* had a higher prevalence of more severe HPE types. Examples of characteristic findings on neuroimaging are shown in Figure 1.

Table 1. Prevalences of HPE types.

HPE type	**Patients with mutations in *ZIC2* (%) (n = 137)**	**Patients with deletion of *ZIC2* (%) (n = 16)**
Alobar	21	38
Semilobar	32	19
Lobar	8	7
MIHV	3	0
Microform	4	0
None	5	0
Unknown	26	38

Table 2. Prevalences of HPE types among probands with known HPE type.

HPE Type	**Patients with mutations in *ZIC2* (%) (n = 83)**	**Patients with deletion of *ZIC2* (%) (n = 10)**

Alobar	33	60
Semilobar	51	30
Lobar	12	10
MIHV	5	0

Clinical Features

Among all individuals with mutations (including both probands and relatives of probands) for whom gender was known, 52% (61/118) were female and 48% (57/118) were male. Among probands for whom gender was known, 51% (43/84) were female and 49% (41/84) were male. There was no statistically significant difference between genders for either all individuals or probands alone.

Patients with recognizable brain anomalies invariably had some degree of neurological impairment. Of 66 families tested, 18 parents were identified as having mutations initially found in their severely-affected children; of those who were subsequently fully examined, only 2 parents were not found to have mild features of microform HPE. The overall penetrance of phenotypic manifestations (including microform HPE) due to mutations in *ZIC2* is estimated to be 96%; the prevalence of brain anomalies is estimated to be 90%.

While many individuals who received a full genetics evaluation had facial dysmorphisms, 67% (39/58) of patients with mutations in *ZIC2* did not display typical HPE facial features such as hypotelorism, flat nasal bridge, cleft lip/palate, or SMCI, features frequently seen in patients with mutations in genes such as *SHH* and *SIX3*[20] . No patients had facial findings at the most severe end of the spectrum, such as cyclopia or synophthalmia, though one patient with semilobar HPE was described as having a

proboscis. A review of photos (figure 2) of available probands (n = 30) with mutations in *ZIC2* revealed a common phenotype in many patients consisting of bitemporal narrowing, upsplanting palpebral fissures, a short nose with anteverted nares, broad and well-demarcated philtrums, and relatively large ears, even accounting for microcephaly (Table 3). Although additional photos were not available for review, a similar facial phenotype was independently described by collaborators (S.M., S.O., CNRS Génétique et Développement, Université de Rennes/ Service de génétique clinique, CHU Hôpital Sud, Rennes, France). On review, this facial phenotype also occurs in previously published patients with mutations in *ZIC2*[16,23]. Facial clefts, ranging from cleft lip and palate to a small unilateral nostril cleft, were described in 10% (7/69), while 17% (12/69) did not have clefts, but had high palates.

Table 3. Description of common dysmorphic features in probands shown in Figure 2.

Patient	HPE type	BN	USPF	FNB	SNAN	BDP	LE	Other	Reference
1	A	+	+	+	+	+		Synophrys	This report
2	A		+	+	+				This report
3	A	+	+		+	+		Tall forehead	This report
4	A	+	+			+			This report
5	A	+	+	+	+	+			[16]
6	A		+				+	Sloping forehead	[16]
7	S		+		+				This report
8	S	+	+	+	+	+			This report
9	S		+		+	+			This report
10	S	+	+	+	+	+	+	Tall, broad forehead	This report
11	S	+				+		Tall, narrow	This report

								head	
12	S		+		+	+	+	Slight synophrys, epicanthal folds, cupid-bow upper lip	This report
13	S		+	+				Broad forehead	This report
14	S	+	+		+		+		This report
15	S		+		+		+		This report
16	S		+		+		+	Synophrys	This report
17	S		+				+	Synophrys	This report
18	S		+	+	+				This report
19	S		+		+		+		This report
20	S	+	+			+	+	Tall forehead	This report
21	S		+						This report
22	S	+	+		+		+	Tall forehead	[23]
23	S	+	+		+	+			[23]
24	S	+	+	+	+			Triangular mouth, myopathic facies	[23]
25	L	+	+	+	+				[23]
26	MIHV		+		+		+		This report
27	MIHV		+		+	+			This report
28	MIHV		+	+		+			[23]
29	Unknown		+		+		+	Sloping forehead	This report
30	Unknown	+	+		+	+		Tall forehead	This report

BN: Bitemporal narrowing; USPF: Upslanting palpebral fissures; FNB: Flat nasal bridge; SNAN: Short nose and/or anteverted nares; BDP: Broad or deep philtrum; LE: Large ears

In terms of neurological defects beyond HPE, 12% (11/93) of individuals had hydrocephalus, and 4% (4/93) were reported as having neural tube defects. Finally, in terms of non-neurological manifestations, 14% had skeletal anomalies, 9% had cardiac anomalies, 7% had renal anomalies, 7% had genital anomalies, 4% had gastrointestinal anomalies, and 4% had pulmonary anomalies (n =76). Five percent had more than 3 congenital anomalies in these systems, including complex congenital heart, renal, and skeletal abnormalities.

Genotypic and functional analysis

The molecular findings among patients with mutations in *ZIC2* have been recently and extensively analyzed[17]. Among kindreds with molecularly-identified mutations, 84% (84/100) were unique. One mutation, which resulted in an alanine expansion and which has been show to result in greatly reduced function, occurred in 11 apparently unrelated kindreds.

Among the 100 unrelated kindreds with molecularly-demonstrated mutations, 38% (38/100) had frameshift mutations, 21% (21/100) had missense mutations, 16% (16/100) were nonsense mutations, 16% (16/100) were in-frame duplications, 5% (5/100) were predicted to result in alternative splicing, 3% (3/100) were in-frame deletions, and 1% (1/100) was an in-frame insertion. 89% (17/19) of the in-frame deletions and duplications occurred in the poly-alanine segment of the gene.

The vast majority (98%) of family-specific mutations were predicted or proven significant loss-of-function. Interestingly, among the very few patients whose mutations were not predicted null, alobar HPE was not observed and 66% (2/3) were inherited, in contrast to the overall estimation that 69% of mutations were *de novo*. There was no

correlation between the type, location, and functional activity conferred by a mutation with the presence of facial dysmorphisms or with HPE severity.

Mutations in *ZIC2* are one of the two most common single-gene causes of non-syndromic HPE (with *SHH*). As patients with *ZIC2* mutations may not have facial dysmorphisms typically associated with HPE, the diagnosis of HPE may not be obvious on clinical encounter. Mutations in *ZIC2* may be an underappreciated cause of HPE, especially in the instance of an early fetal demise when high-quality brain imaging or pathologic analysis is not available.

However, our analysis of this large cohort of patients with mutations in *ZIC2* reveals several unique features resulting from mutations in this gene which distinguish the patients described here from patients with mutations in other HPE-associated genes. First, many patients with mutations in *ZIC2* have a subtle but distinct dysmorphic facial phenotype which may help aid diagnosis. This facial appearance is unique among patients with HPE, and has not been seen in patients with HPE due to other genetic etiologies. Second, unlike other genes associated with HPE, the majority of mutations occur *de novo*. Our data suggests the presence of at least 4 families in which germline mosaicism seems to be causative of HPE in a child, which has important implications for genetic counseling. Parents who test negative for *ZIC2* mutations through analysis of peripheral blood may still be at risk for having other affected children.

Third, along these lines, we did not identify any large pedigrees in which numerous individuals from multiple generations were identified, which is not the case for the other common HPE-associated genes such as *SHH* or *SIX3*. This could imply that

mutations in *ZIC2* are less likely to result in mildly-affected individuals than mutations in other HPE-associated genes. Since *ZIC2* mutations occur relatively frequently in non-syndromic HPE, this would further imply that the mutation rate for these mutations is higher than, for example, mutations in *SIX3*, which are overall less frequent, but occur more often in large kindreds with multiple affected generations[20]. However, the high penetrance and relatively severe findings may bring individuals to clinical attention earlier, resulting in ascertainment bias.

Finally, laboratories and clinicians must be aware of the importance of functional data in order to characterize mutations and to inform counseling of affected families; we know specifically of certain repeat variants in *ZIC2* resulting in different numbers of histidine repeats, which have previously been thought to be pathogenic, but on later family analysis, are now thought to be polymorphisms which may be common in ethnicities not originally part of control populations[17].

One shortcoming of this report is that the available retrospective collection of clinical data was not uniform. For this reason, it is likely that we underestimate the prevalence of many of the findings (such as neural tube defects and other congenital anomalies). Despite the challenges synthesizing the data, the availability of a large cohort of patients with mutations affecting the same gene greatly enriches our understanding of HPE in general and *ZIC2* in particular. This study demonstrates the existence of a previously unnoticed *ZIC2*-specific phenotype, and highlights the importance of a comprehensive and collaborative approach to study HPE and other complex genetic disorders.

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Figure Legends

Figure 1. Characteristic findings on neuroimaging. a: Alobar HPE with shunt in place; b,c: semilobar HPE with large dorsal cyst; d: semilobar HPE without dorsal cyst; e,f: MIHV-type HPE

Figure 2. Patients with mutations in *ZIC2*, arranged by HPE type. Note *ZIC2*-specific facial findings, consisting of bitemporal narrowing, upslanting palpebral fissures, flat nasal bridge and a short nose with upturned nares, a broad and/or deep philtrum, and large ears.