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

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ABSTRACT

Background Holoprosencephaly (HPE), the most common malformation of the human forebrain, may be due to mutations in genes associated with non-syndromic HPE. Mutations in *ZIC2*, located on chromosome 13q32, are a common cause of non-syndromic, non-chromosomal HPE.

Objective To characterise genetic and clinical findings in patients with *ZIC2* mutations.

Methods Through the National Institutes of Health and collaborating centres, DNA from approximately 1200 individuals with HPE spectrum disorders was analysed for sequence variations in *ZIC2*. Clinical details were examined and all other known cases of mutations in *ZIC2* were included through a literature search.

Results By direct sequencing of DNA samples of an unselected group of unrelated patients with HPE in our NIH laboratory, *ZIC2* mutations were found in 8.4% (49/582) of probands. A total of 157 individuals from 119 unrelated kindreds are described, including 141 patients with intragenic sequence determined mutations in *ZIC2*. Only 39/157 patients have previously been clinically described. Unlike HPE due to mutations in other genes, most mutations occur de novo and the distribution of HPE types differs significantly from that of non-*ZIC2* related HPE. Evidence is presented for the presence of a novel facial phenotype which includes bitemporal narrowing, upslanting palpebral fissures, a short nose with anteverted nares, a broad and well demarcated philtrum, and large ears.

Conclusions HPE due to *ZIC2* mutations is distinct from that due to mutations in other genes. This may shed light on the mechanisms involved in formation of the forebrain and face and will help direct genetic counselling and diagnostic strategies.

INTRODUCTION

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 categorised by the degree of forebrain separation into alobar, semilobar, and lobar types, from most to least severe. More recently, middle interhemispheric variant (MIHV) HPE has also been described, which includes failed separation of only 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 10–40% alobar, 43–45% semilobar, and 17–33% lobar HPE (Muenke Lab, unpublished data, 2010).[7 8]

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, and a single maxillary central incisor (SMCI) without appreciable central nervous system (CNS) anomalies on conventional neuroimaging. These individuals are often identified due to the presence of a severely affected relative.[6 9 10]

HPE is aetiologically 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 7 9 11–13] 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 14]

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 analyses of patients with HPE identified mutations in *ZIC2*.[15–17] *ZIC2* mutations have 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*, though a more accurate estimate is likely to be at least double that.[6 18]

ZIC2 encodes a transcription factor that 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.[19 20] 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.[21] 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 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.[18 20 22 23] Of note, it has been suggested that mutations in *ZIC2* may cause HPE brain findings, but often do not result in facial features typical of HPE due to mutations in other genes—in other words, the face would not 'predict the brain'.[17 24]

Here we present clinical and genetic data on all known individuals with mutations in *ZIC2*, approximately half of whom were identified through our laboratory at the National Institutes of Health (NIH), and over three quarters of whom have not been previously clinically described. We also present data on individuals with deletions of the *ZIC2* locus ascertained by multiplex ligation dependent probe amplification (MLPA) and fluorescence in situ hybridisation (FISH), chromosome analysis, or by oligonucleotide array comparative genomic hybridisation (aCGH). Through this comprehensive evaluation, we can identify specific characteristics of these individuals that can differentiate patients with HPE due to *ZIC2* mutations from patients with HPE due to other genetic causes.

METHODS

Patient recruitment, mutation screening, and clinical assessments

Blood samples from approximately 600 individuals with HPE spectrum disorders and their relatives were collected over 18 years in our laboratory at the NIH. These samples were analysed for potential sequence variations in the *ZIC2* gene under our National Human Genome Research Institute/NIH Institutional Review Board (IRB) approved brain research protocol (with appropriate consent). A strategy for screening the *ZIC2* gene has previously been described.[18]

Approximately 600 additional probands with HPE were screened through collaborating centres, for a total of approximately 1200 probands with HPE spectrum anomalies. This total cohort includes deceased fetuses (of note, the approximately 600 patients included in the NIH cohort does not include deceased fetuses), live born infants, and currently living patients. After mutation was identified in a proband, additional individuals were identified through testing of relatives. The analysis of clinical characteristics was performed retrospectively; the quality of available clinical information was highly variable.

In terms of NIH patients, before 2006, referring clinicians (which include geneticists, neurologists, obstetricians, and pathologists) were asked to send samples with available clinical data, including clinical summaries, photos, and neuroimaging. Starting in 2006, referring clinicians additionally filled out a standardised, brief clinical checklist describing clinical findings, family history, and risk factors. In the process of reviewing information for this analysis, many referring clinicians were recontacted in order to request additional data. Four patients were seen at the NIH for a comprehensive evaluation.

In terms of patients who were not part of the NIH cohort, information was obtained through collaborators who sent de-identified clinical and laboratory data: Laboratoire de Génétique Moléculaire (Rennes, France); Center for and Department of Human Genetics (Regensburg, Germany); GeneDx (Gaithersburg, Maryland, USA); Maastricht University Medical Centre (Maastricht, The Netherlands). Collaborating centres shared (with appropriate consent) available clinical data, typically in the form of a narrative summary, photos and neuroimaging results.

All patients on whom craniofacial data are presented were assessed in person by clinical dysmorphologists. Thirty probands had photos available for review, while detailed physical examination assessments performed by clinical dysmorphologist were available for 29 additional probands. All HPE types were identified by neuroimaging, performed by ultrasound (in the majority of fetal cases), CT, MRI, or by pathological study. MRI was available in approximately half of cases with identified HPE type, with a bias for more recently ascertained cases.

Literature review of reported cases of holoprosencephaly spectrum disorders due to mutations in *ZIC2*

A Medline search was conducted to find previously reported cases of holoprosencephaly due to mutations in *ZIC2*. The key words and search terms included '*ZIC2*', 'holoprosencephaly', 'HPE', '13q', and '13q32'. References were also obtained from articles found through the literature search. As loci near *ZIC2* may contribute to brain malformations and there have been numerous reported cases of deletions of 13q with unreported clinical and genetic characterisations, only cases with 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*, 1999; 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; Paulussen *et al*, 2008, *Eur Soc of Hum Genet*, abstract; Roessler *et al*, 2009; Quélin *et al*, 2009.[15–18 24–34]

Statistical analysis

In the descriptions below, unless otherwise stated, results refer only to individuals with intragenic sequence determined mutations in *ZIC2*, not patients with large genomic imbalances. Patients with large genomic imbalances were not included in

most statistical analyses due to the relatively low numbers and because the testing method differed among patients (including traditional cytogenetic analysis, FISH testing, and aCGH), potentially invalidating comparisons.

Denominators differ among findings, as the prevalence of each phenotypic manifestation was calculated only where data were available for that specific finding (table 1). χ^2 and Fisher's exact tests were used to determine statistical differences between patient groups.

RESULTS

Patients

We describe a total of 157 patients, including 141 patients from 103 unrelated kindreds with sequence determined mutations in *ZIC2*, seven patients with deletions of *ZIC2* ascertained by FISH testing or MLPA, and nine patients with deletions of *ZIC2* ascertained by chromosome analysis or by oligonucleotide aCGH. While the majority of these mutations have been reported, only 25% (39/157) have previously been clinically described. Of the 157 patients, 77 patients were identified at the NIH, 72 were identified through collaborating centres, and eight patients were identified through a literature search (all eight of whom were patients with deletions of the *ZIC2* locus as part of a larger genetic imbalance).[15–17 24–34]

By direct sequencing of DNA samples of an unselected group of unrelated patients with HPE in our laboratory at the NIH, 8.4% (49/582) have mutations in *ZIC2*. Additional cases were initially ascertained through screening methodology, including screening methods involving single strand conformational polymorphism (SSCP) analysis and denaturing high performance liquid chromatography (dHPLC). Multiple international testing centres additionally contributed cases as described above (Methods). A summary of all patients is presented in table 2.

Inheritance

Among probands in whom parents were available for testing (65/103 families), mutations were found to be de novo in 72%, maternally inherited in 18%, and paternally inherited in 9% of patients. There were no kindreds in which mutations or affected individuals were identified in more than two generations. However, in five 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 dropout or, more likely, germline mosaicism.

HPE type

Prevalences of HPE types are presented in table 3. Examples of characteristic findings on neuroimaging are shown in figure 1.

Table 1 Denominators used to calculate prevalence of findings in individuals with intragenic sequence determined mutations in *ZIC2*

	Probands	All affected individuals*
Total patients	103	141
Gender	90	127
Holoprosencephaly (HPE) type	86	105
Structural neurological findings†	81	92
Inheritance	65	85
Extra-neurological findings	64	76
Facial phenotype‡	59	59

*Including probands and relatives.
†In addition to HPE.
‡30 photos, 29 full facial descriptions.

For patients with intragenic sequence determined mutations, the distribution of HPE types is not equal, with alobar and semilobar HPE significantly overrepresented ($\chi^2_{(2)}$=23.65, p<0.0001). MIHV was not included in the analysis due to paucity of cases. Due to the relatively few cases, the group of patients with larger genomic imbalances, including deletion of *ZIC2*, was not analysed.

We compared the distribution of HPE types in patients with intragenic sequence determined mutations in *ZIC2* to two previous studies describing the prevalence of the major HPE types, as well as to a group of HPE probands ascertained from samples sent to the NIH for clinical testing in an approximately 3 year period after the establishment of a reference laboratory (table 4).[7 8] The groups described by Lazaro *et al* (2004) and the NIH groups are likely most similar to our cohort by the fact that all three groups had non-chromosomal, non-syndromic HPE.[7] The cohort described by Orioli *et al* (2007), on the other hand, may include some patients with chromosomal anomalies, and also included only patients who survived to birth.[8] There was a significantly different distribution of HPE types in our cohort of patients with intragenic *ZIC2* mutations versus those described by Lazaro *et al* (2004) and ascertained through our general NIH HPE cohort.[7] There was not a statistically significant difference compared to the Orioli *et al* (2007) group, though this latter cohort does not appear well matched with our *ZIC2* cohort.[8]

Clinical features

Among all individuals with mutations (including both probands and relatives of probands) for whom gender was known, 50% were female and 50% were male. Among probands for whom gender was known, 51% were female and 49% were male.

Patients with recognisable brain anomalies invariably had some degree of neurological impairment. Of 65 families tested, 18 parents were identified as having mutations initially found in their severely affected children; of the eight parents who had mutations (not germline mutations) who were fully examined, only two parents were not found to have mild features of microform HPE. The overall penetrance of phenotypic manifestations (including alobar, semilobar, lobar, MIHV, and microform HPE, as well as HPE of unknown type) due to intragenic mutations in *ZIC2* is estimated to be 93%; the prevalence of structural brain anomalies consistent with a diagnosis of frank HPE (by conventional neuroimaging or pathology) is estimated to be 88% of patients with intragenic mutations.

Among 59 patients for whom information was available, at least 67% (40/59) did not display typical HPE facial features such as the combination of hypotelorism, midface hypoplasia with flat nasal bridge, cleft lip/palate, and SMCI, features frequently seen in patients with mutations in genes such as *SHH* and *SIX3*.[21] While 33% of patients (19/59) were reported as having facial characteristics commonly described in HPE (as above), none of these latter patients had photographs available for review, and were only described in clinical summaries provided by referring clinicians. In other words, none of the 30 available photos show facial features in which patients with mutations in *ZIC2* have typical HPE craniofacial manifestations, but some clinicians describe more typical HPE facial features in written summaries. Additionally, no patients had facial findings at the most severe end of the spectrum, such as cyclopia, synophthalmia, or a proboscis. As anthropometric measurements were not uniformly available, detailed calculations as to the prevalence of certain features were not attempted.

Table 2 All known patients with mutations affecting *ZIC2*

Patient*	HPE type	Gender	Case Status	Inheritance	DNA Alteration	Predicted protein alteration in ZIC2	Functional activity	Reference
1	U	F	P	De novo	c.21delG	p.Q8SfsX33	Predicted null	18 24
2	S	M	P	De novo	c.81_86delGGCGGCinsTCGGT	p.A28RfsX13	Predicted null	18
3a	S	F	P	Maternal	c.107A→C (assumed)	p.Q36P (assumed)	170%	18 29 31
3b	Mic	F	Mother	Unknown	c.107A→C	p.Q36P	170%	18 29 31
4a	L	F	P	Paternal	c.109G→A	p.D37N	Unknown	18
4b	N	M	Father	Unknown	c.109G→A	p.D37N	Unknown	18
5	S	F	P	De novo	c.129_184dup56	p.L62Rfs175	Predicted null	18
6	S	F	P	De novo	c.136C→T	p.Q46X	Predicted null	18 31
7	A	F	P	De novo	c.172G→T	p.G58X	Predicted null	18 31
8	S	F	P	De novo	c.177ins56	p.F60QfsX176	Predicted null	17 18
9a	S	F	P	Maternal	c.191dupC	p.A66RfsX301	Predicted null	18
9b	L	M	Brother	Maternal	c.191dupC	p.A66RfsX301	Predicted null	18
9c	Mic	F	Mother	Unknown	c.191dupC	p.A66RfsX301	Predicted null	18
10	L	M	P	De novo	c.217C→T	p.Q73X	Predicted null	18
11	A	F	P	Unknown	c.217delC	p.Q73Rfs145	Predicted null	18
12	A	M	P	De novo	c.367delA	p.S123AfsX95	Predicted null	18
13	A	M	P	De novo	c.382G→A	p.D128N	Unknown	18
14a	A	M	P	Paternal	c.386_392delCGGCGCC	p.S129WfsX87	Predicted null	18
14b	S	M	Brother	Paternal	c.386_392delCGGCGCC (assumed)	p.S129WfsX87 (assumed)	Predicted null	18
14c	Mic	M	Father	Unknown	c.386_392delCGGCGCC (assumed)	p.S129WfsX87 (assumed)	Predicted null	18
15	U	U	P	Unknown	C.392_398del7	p.G133SfsX83	Predicted null	18
16a	L	F	P	Maternal	c.454_455delinsTT	p.D152F	60%	18 24 31
16b	N	F	Mother	Unknown	c.454_455delinsTT	p.D152F	60%	18 24 31
17	L	M	P	Germline	c.479delC	p.P160RfsX58	Predicted null	18
18	A	M	P	De novo	c.490G→T	p.E164X	Predicted null	18
19a	S	M	P	Unknown (assumed germline mosaicism)	c.557_572dup16	p.E192GfsX180	Predicted null	18
19b	S	F	Sister	Unknown (assumed germline mosaicism)	c.557_572dup16	p.E192GfsX180	Predicted null	18
20a	U	F	P (triplet)	Unknown	c.577delC	p.Q193NfsX25	Predicted null	18
20b	U	F	Sister (triplet)	Unknown	c.577delC	p.Q193NfsX25	Predicted null	18
21	A	M	P	De novo	c.582C→A	p.Y194X	Predicted null	18
22	S	F	P	De novo	c.612delC	p.Y205TfsX13	Predicted null	18
23	S	M	P	Unknown	c.622GC→TT	p.A208L	Unknown	This report
24	A	M	P	Unknown	c.659delA	p.N220TfsX4	Predicted null	18
25a	A	F	P	Maternal	c.665_676dup12	p.G222_M225dup	Unknown	18
25b	U	F	Mother	Unknown	c.665_676dup12	p.G222_M225dup	Unknown	18
26	S	F	P	De novo	c.748C→T	p.Q250X	Predicted null	18
27	A	M	P	De novo	c.779G→A	p.W260X	Predicted null	18
28a	S	M	P	Unknown	c.793C→T	p.Q265X	Predicted null	18
28b	U	F	Sister	Unknown	c.793C→T	p.Q265X	Predicted null	18
29	A	M	P	De novo	c.797_801del	p.L266QfsX99	Predicted null	This report
30a	MIHV	F	P	Maternal (assumed germline mosaicism)	c.808_809ins17	p.K270TfsX2	Predicted null	18 31
30b	A	M	Brother	Maternal (assumed germline mosaicism)	c.808_809ins17 (assumed)	p.K270TfsX2 (assumed)	Predicted null	18 31
30c	U	M	Brother	Maternal (assumed germline mosaicism)	c.808_809ins17 (assumed)	p.K270TfsX2 (assumed)	Predicted null	18 31
30d	N	F	Mother	Unknown	c.808_809ins17 (assumed germline mosaicism)	p.K270TfsX2 (assumed germline mosaicism)	Predicted null	18 31
31	A	M	P	Unknown	c.815G→A and c. 974 G→T	p.S272N p.R325L	Predicted null (for both mutations)	18
32	U	U	P	Unknown	c.825_826delAA	p.K275NfsX91	Predicted null	18
33	U	F	P	Unknown	p.829_830dupTT	p.T279AfsX7	Predicted null	18
34	A	M	P	De novo	c.856C→T	p.H286Y	Predicted null	18
35	U	U	P	Unknown	p.857A→T	p.H286L	Predicted null	18
36	A	F	P	Unknown	c.858C→G	p.H286Q	Predicted null	18
37	S	U	P	De novo	c.862_863delTC	p.S288GfsX78	Predicted null	18 28
38a	L	M	P	Paternal	c.871C→T	p.H291Y	Predicted null	18
38b	Mic	M	Father	Unknown	c.871C→T	p.H291Y	Predicted null	18

Continued

Table 2 Continued

Patient*	HPE type	Gender	Case Status	Inheritance	DNA Alteration	Predicted protein alteration in ZIC2	Functional activity	Reference
39	S	M	P	Unknown	c.910T→A (Also *SIX3*: c.850G→C)	p.W304R (Also SIX3: p.A284P)	Predicted null	18
40	A	F	P	Unknown	c.912G→A	p.W304X	Predicted null	18
41	U	M	P	Unknown	c.912G→A	p.W304X	Predicted null	18
42	L	F	P	De novo	c.928G→T	p.E310X	Predicted null	18
43	S	F	P	De novo	c.932delG	p.G311AfsX102	Predicted null	18 24
44	A	F	P	Unknown	c.941T→G	P.F314C	Predicted null	18
45	S	F	P	De novo	c.973C→A	p.R325S	Predicted null	18
46	U	U	P	Unknown	c.974G→T	p.R325L	Predicted null	18
47	U	M	P	De novo	c.979C→T	p.H327Y	Predicted null	18
48	S	M	P	De novo	c.994_1005dup	p.C335_P338dup	Predicted loss-of-function	This report
49	A	F	P	De novo	c.1004G→T	p.C335F	Predicted null	18
50	S	F	P	De novo	c.1025_1026delAA	p.K342SfsX24	Predicted null	18 31
51	S	U	P	De novo	c. 1031_1032 delTC	p.F344CfsX22	Predicted null	18 24
52	A	F	P	De novo	c.1040_1046del	pE348SfsX63	Predicted null	17 18 24
53	S	M	P	Unknown	c.1051A→T	p.K351X	Predicted null	18
54	S	F	P	De novo	c.1052_1053insAA AGGTTCACAC AGAACCTCAA	p.K351_I352Ins7	Predicted null	18
55	A	M	P	De novo	c.1075+2T→A (IVS+1T→A)	p.G359fsX62	Predicted null	18
56	U	F	P	Unknown	c.1076-1G→A (IVS1-1G→A)	Alternative splicing	Predicted null	18
57	U	M	P	De novo	c.1076-1G→A (IVS1-1G→A)	Alternative splicing	Predicted null	18
58	S	M	P	Unknown	c.1076-1G→A (IVS1-1G→A)	Alternative splicing	Predicted null	18
59	S	M	P	Unknown	c.1076-1G→A (IVS1-1G→A)	Alternative splicing	Predicted null	18
60	S	F	P	De novo	c.1090C→T	p.Q364X	Predicted null	18
61a	S	M	P (twin)	De novo	c.1091_1092delAG	p.Q364LfsX2	Predicted null	18 24 31
61b	U	M	Twin	De novo	c.1091_1092delAG	p.Q364LfsX2	Predicted null	18 24 31
62	S	M	P	Unknown	c.1095_1096delTG	p.C365X	Predicted null	18
63a	S	F	P	Maternal	c.1095_1096delTG	p.C365X	Predicted null	18
63b	U	M	Half-sibling	Maternal (assumed)	c.1095_1096delTG (assumed)	p.C365X (assumed)	Predicted null	18
63c	U	F	Mother	Unknown	c.1095_1096delTG	p.C365X	Predicted null	18
64	S	M	P	De novo	c.1097_1098delAG	p.E366Vfs2	Predicted null	18 24 31
65	U	U	P	Unknown	c.1118G→C	p.R373P	Predicted null	18
66	S	M	P	Unknown	c.1119_1120delCT	p.F374RfsX17	Predicted null	18
67	U	U	P	Unknown	c.1204T→A	p.Y402N	Predicted null	18
68a	S	F	P	Maternal	c.1206C→G	p.Y402X	Predicted null	18
68b	U	M	Brother	Maternal	c.1206C→G	p.Y402X*	Predicted null	18
68c	Mic	F	Mother	Unknown	c.1206C→G	p.Y402X	Predicted null	18
69a	S	F	P	Maternal	c.1208C→A	p.T403K	Predicted null	18
69b	U	F	Half-sister	Maternal	c.1208C→A (assumed)	p.T403K (assumed)	Predicted null	18
69c	U	F	Half-sister	Maternal	c.1208C→A (assumed)	p.T403K (assumed)	Predicted null	18
69d	U	F	Mother	Unknown	c.1208C→A (assumed)	p.T403K (assumed)	Predicted null	18
70	S	M	P	Unknown	c.1211A→G	p.H404R	Predicted null	18
71	S	F	P	Unknown	c.1225C→T	p.R409W	Predicted null	18
72	U	U	P	Unknown	c.1239+1G→C (IVS1-1G→A)	Possible inclusion of intron 2 codons or alternative splicing	Predicted null	18
73	U	U	P	Unknown	c.1240-2A→G (IVS2-2A→G)	Alternative splicing	Predicted null	18
74	L	M	P	Unknown	c.1245T→G	p.H415Q	Predicted null	18
75	S	F	P	Unknown	c.1277delC	p.P426RfsX129	Predicted null	18
76	A	M	P	De novo	c.1313dupC	p.L440AfsX90	Predicted null	17 18
77	L	F	P	De novo	c.1323dupG	p.S442Vfs88	2%	18 24 29
78	A	M	P	Unknown	c.1329delC	p.S444AfsX111	Predicted null	This report
79	MIHV	M	P	De novo	c.1330_1365del	p.444_455del	60%	18 24 29
80	S	M	P	Unknown	c.1366_1395dup30	p.A456_465dup	Unknown	This report
81	MIHV	M	P	Unknown	c.1366_1395dup30	p.A456_465dup	Unknown	This report
82a	A	M	P	Paternal (assumed germline mosaicism)	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	17 18 24
82b	A	F	Sister	Paternal (assumed germline mosaicism)	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	17 18 24
82c	U	M	Paternal half-sister	Paternal (assumed germline mosaicism)	c.1377_1406dup30 (assumed)	p.A461_470dup (assumed)	5% with reduced DNA binding	17 18 24

Continued

Table 2 Continued

Patient*	HPE type	Gender	Case Status	Inheritance	DNA Alteration	Predicted protein alteration in ZIC2	Functional activity	Reference
82d	U	F	Paternal half-sister	Paternal (assumed germline mosaicism)	c.1377_1406dup30 (assumed)	p.A461_470dup (assumed)	5% with reduced DNA binding	17 18 24
82e	Mic	M	Brother	Paternal (assumed germline mosaicism)	c.1377_1406dup30 (assumed)	p.A461_470dup (assumed)	5% with reduced DNA binding	17 18 24
82f	S	F	Sister	Paternal (assumed germline mosaicism)	c.1377_1406dup30 (assumed)	p.A461_470dup (assumed)	5% with reduced DNA binding	17 18 24
82g	N	M	Father	De novo	c.1377_1406dup30 (assumed germline mosacisim)	p.A461_470dup (assumed germline mosaicism)	5% with reduced DNA binding	17 18 24
83	U	F	P	Unknown	c.1377_1406dup30 (also *SHH*: c. 869G→A)	p.A461_470dup (also SHH: p.G290D)	5% with reduced DNA binding	18 26
84	S	F	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	This report
85a	S	U	P	Maternal	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18
85b	U	F	Mother	Unknown	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18
86a	MIHV	F	P	Paternal	c.1377_1406dup30; Also sib with *FOXH1*: c.1062delT	p.A461_470dup; Also sib with FOXH1: FS in COOH terminus	5% with reduced DNA binding	18 24
86b	U	M	Brother	Paternal	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18 24
86c	N	M	Father	"Mosaic carrier"	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18 24
87	S	F	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18 34
88	A	F	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18
89	S	M	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18 24
90	L	M	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	18
91	S	F	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	This report
92	A	F	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	This report
93	MIHV	M	P	De novo	c.1377_1406dup30	p.A461_470dup	5% with reduced DNA binding	This report
94a	L	F	P	Paternal	c.1377_1406del	p.A456_465del	Predicted null	18
94b	U	M	Father	Unknown	c.1377_1406del	p.A456_465del	Predicted null	18
95a	A	M	P	Maternal	c.1392_1406del	p.A461_465del	Predicted null	18
95b	N	F	Mother	Unknown	c.1392_1406del	p.A461_465del	Predicted null	18
96a	S	M	P	Maternal	c.1401_1406dup	p.A469_470dup	Unknown	This report
96b	U	U	Sibling	Maternal	c.1401_1406dup	p.A469_470dup	Unknown	This report
96c	N	M	Mother	Unknown	c.1401_1406dup	p.A469_470dup	Unknown	This report
97	S	M	P	De novo	c.1420_1427dup; c.1428_1433delinsCG	p.G477CfsX54	Predicted null	18
98	S	F	P	De novo	c.1437_1441del	p.S480QfsX48	Predicted null	This report
99	S	F	P	De novo	c.1445_1461del17	p.S482RfsX42	Predicted null	18
100	A	U	P	Unknown	c.1452_1456delCGCGG	p.A485RfsX43	Predicted null	18
101	S	M	P	Unknown	c.1455_1461delinsCG	p.G487LfsX41	Predicted null	18
102	S	M	P	De novo	c.1508_1520delGCGGC GGGGGCGG	p.G503AfsX48	Predicted null	18
103a	U	U	P	Maternal	c.1559delA	p.H520PfsX35	Predicted null	18
103b	U	M	Brother	Maternal	c.1559delA	p.H520PfsX35	Predicted null	18
103c	U	F	Mother	Unknown	c.1559delA	p.H520PfsX35	Predicted null	18
104	S	F	P	Unknown	Gene deletion	N/A	Predicted null	34
105	S	M	P	De novo	Gene deletion	N/A	Predicted null	This report
106	L	F	P	De novo	Gene deletion	N/A	Predicted null	This report
107	S	M	P	De novo	Gene deletion	N/A	Predicted null	This report
108	A	F	P	De novo	Gene deletion	N/A	Predicted null	This report
109	A	F	P	De novo	Gene deletion	N/A	Predicted null	This report
110	U	M	P	De novo	Gene deletion	N/A	Predicted null	This report
111	U	F	P	U	del13(q31.1-13qter)	N/A	Predicted null	33
112	U	M	P	U	del13(q31.1-13qter)	N/A	Predicted null	33
113	U	M	P	U	del13(q31.3 -13q33.1)	N/A	Predicted null	33

Continued

Table 2 Continued

Patient*	HPE type	Gender	Case Status	Inheritance	DNA Alteration	Predicted protein alteration in ZIC2	Functional activity	Reference
114	U	M	P	U	del13(q32.3)	N/A	Predicted null	33
115	A	F	P	U	del13(q32q34)	N/A	Predicted null	32
116	A	F	P	Maternal	i(13)(q10)	N/A	Predicted null	25
117	U	U	P	U	del(13)(q22qter)	N/A	Predicted null	16
118	A	M	P	U	del(13)(q22qter)	N/A	Predicted null	27
119	U	M	P	U	del(13)(q31qter)	N/A	Predicted null	30

*Each kindred is listed by a separate number; individuals within a kindred are each assigned a separate letter.
A, alobar; F, female; L, lobar; M, male; Mic: microform; MIHV, middle interhemispheric variant; N, none; P, proband; S, semilobar; U, unknown.

Independent reviews (by KR, BS, MM) of photos of available probands with mutations in *ZIC2* revealed a common phenotype consisting of bitemporal narrowing (53%), upslanting palpebral fissures (97%), a flat nasal bridge (33%), a short nose with anteverted nares (73%), a broad and deep philtrum (43%), and the subjective appearance of relatively large ears (37%) (figure 2, table 5). All photos reviewed showed evidence of this common facial phenotype, and none had facial features notably similar to those of patients with HPE due to mutations in other genes. Prevalences of facial findings with data sufficient for comparison are presented in table 6 compared to a cohort with mutations in *SIX3*.

Although additional photos were not available for review, a similar facial phenotype was independently described by collaborators (SM, SO, personal communication). On review, this facial phenotype also occurs in previously published patients with mutations in *ZIC2*.[17 24] Facial clefts, ranging from cleft lip and palate to a small unilateral nostril cleft, were described in 10%, while 17% did not have clefts, but had high palates. Facial clefts in patients with intragenic *ZIC2* mutations are approximately a third as common as in other cohorts with non-*ZIC2* related HPE.[8 21]

In terms of neurological defects, in addition to HPE in patients with intragenic sequence determined mutations (ie, not including patients with whole gene deletions or large cytogenetic imbalances), 12% of individuals had hydrocephalus, and 4% 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. Five per cent had more than three congenital anomalies in these systems, including complex congenital cardiac, renal, and skeletal abnormalities. We present comparisons to the only other large cohort of patients with HPE due to mutations in a single gene (*SIX3*) and to a cohort of patients with non-syndromic HPE (table 6). In this latter comparison, the category of non-syndromic HPE includes patients in whom HPE does not occur in the context of a broader syndrome, but it is important to realise that patients with non-syndromic HPE may present with findings that extend beyond the traditional craniofacial and structural brain anomalies most often recognised as the classic manifestations of autosomal dominant monogenic HPE.[21] While not all features were described in the three cohorts, the comparisons do show statistical support that patients with mutations in *ZIC2* appear to have a unique facial phenotype. Additionally, this comparison shows that patients with mutations in *ZIC2* have overall similar rates of extra-neuronal manifestations to patients with *SIX3* mutations in contrast to a cohort of patients with non-syndromic HPE, although skeletal manifestations appear more frequent in patients with mutations in *ZIC2* than in *SIX3* related HPE.[8 21]

Table 3 Prevalences of holoprosencephaly (HPE) types

HPE type	Patients with mutations in *ZIC2*, n (%)	Probands with mutations in *ZIC2*, n (%)	Patients with deletions of *ZIC2*, n (%)
Alobar	28 (20)	26 (25)	5 (31)
Semilobar	48 (34)	45 (44)	3 (19)
Lobar	11 (8)	10 (10)	1 (6)
MIHV	5 (4)	5 (5)	0 (0)
Microform	6 (4)	0	0 (0)
None	7 (5)	0	0 (0)
Unknown	36 (26)	17 (17)	7 (44)
Total	141	103	16

MIHV, middle interhemispheric variant.

Genotypic and functional analysis

The molecular findings among patients with mutations in *ZIC2* have been recently and extensively analysed.[18] Among kindreds with intragenic sequence determined mutations, 81% were unique. One mutation, which resulted in an alanine expansion and which has been shown to result in greatly reduced function, occurred in 12 apparently unrelated kindreds.

Among the 103 unrelated kindreds with intragenic sequence determined mutations, 37% had frameshift mutations, 21% had missense mutations, 17% were in-frame duplications or insertions, 16% had nonsense mutations, 6% were predicted to result in alternative splicing, and 3% were in-frame deletions; 89% of the in-frame deletions and duplications occurred in the polyalanine 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 (kindreds 3, 16, and 79) whose mutations were shown by functional analysis not to be null, alobar HPE was not observed and 66% (2/3) were inherited, in contrast to the overall estimation that 72% of mutations in probands occur de novo. Due to the low number of kindreds with mutations not shown to be null and the fact that equivalent functional analyses have not been performed for most mutations, statistical calculations involving the latter observation were not attempted. The overall rate of de novo mutations in *ZIC2* is in stark contrast to patients with HPE due to mutations in *SHH* or *SIX3*, in which the de novo mutation rate is estimated to be 10–30%, and 14%, respectively (Muenke lab, unpublished data, 2010).[21]

In our analysis, we did not include previously reported variants in *ZIC2* resulting in different numbers of histidine repeats, which had been thought to be pathogenic, but on later pedigree analysis, are now thought to be polymorphisms that may be common in ethnicities not originally part of control populations.[18 35 36]

DISCUSSION

Mutations in *ZIC2* are one of the two most common single gene causes of non-syndromic HPE. We show that patients with *ZIC2*

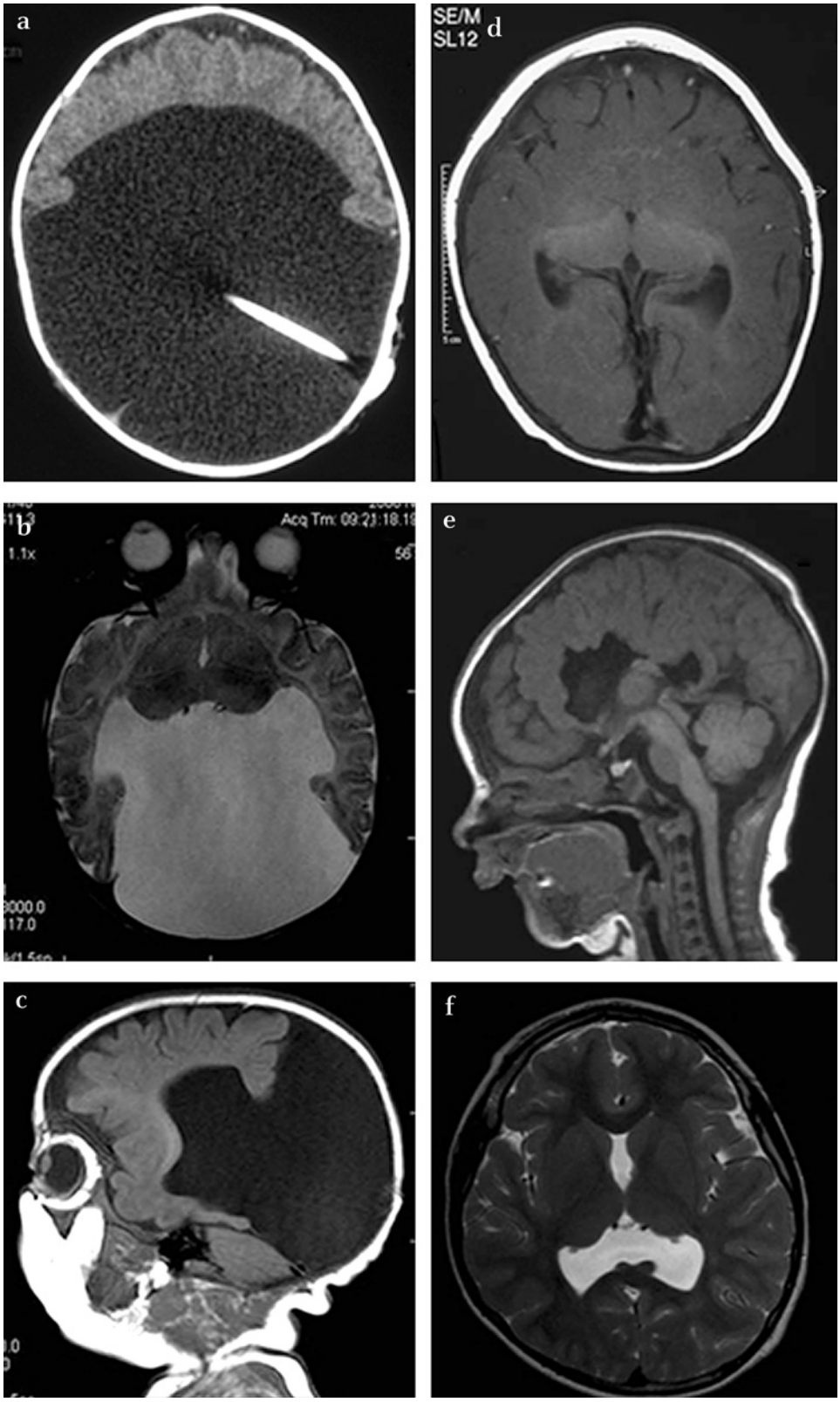

Figure 1 Characteristic findings on neuroimaging. (A) Alobar holoprosencephaly (HPE), with shunt in place. (B, C) Semilobar HPE with large dorsal cyst. (D) Semilobar HPE without dorsal cyst. (E, F) Middle interhemispheric variant (MIHV) type HPE.

mutations do not typically have facial dysmorphisms standardly associated with HPE. HPE due to *ZIC2* mutations could be underappreciated, as HPE is not diagnosed in some patients with *ZIC2* mutations due to the absence of facial dysmorphisms leading to a diagnosis of HPE.

Further, our analysis of this large cohort reveals several unique features which distinguishes *ZIC2* related HPE from HPE due to other causes. First, the recognition that many patients with mutations in *ZIC2* have a subtle but distinct facial phenotype may help aid diagnosis. This facial appearance has not been described in patients with HPE resulting from other genetic aetiologies. The data in our cohort may be biased because only some patients survived long enough for photographs to be taken. However, the fact that no photographs demonstrated a combination of features

Table 4 Comparison of holoprosencephaly (HPE) type distribution among the three 'classic' HPE types for the patients with intragenic *ZIC2* mutations, as well as two sources from the literature and a source obtained from our database: Lazaro *et al* (2004) described a cohort of both living patients and deceased fetuses with non-chromosomal, non-syndromic HPE, while Orioli *et al* (2007) describes a cohort of patients born with HPE.[7 8] We also compare our cohort of patients with mutations in *ZIC2* with a cohort of prospectively ascertained probands with non-chromosomal, non-syndromic HPE whose samples were sent to the National Institutes of Health (NIH) over approximately a 3 year period. Due to low prevalence, middle interhemispheric variant (MIHV) was not considered

	Intragenic ZIC2 mutations n (%)	Lazaro *et al*[7] n (%)	Orioli and Castilla[8] n (%)	NIH n (%)
Alobar	28 (32)	15 (22)	33 (40)	10 (13)
Semilobar	48 (55)	31 (45)	36 (43)	45 (6)
Lobar	11 (13)	23 (33)	14 (17)	20 (27)
Total	87	69	83	75
Comparison vs *ZIC2* cohort		$\chi^2_{(2)}$=9.88, p=0.0077	$\chi^2_{(2)}$=2.39, p=0.30	$\chi^2_{(2)}$=10.4, p=0.0055

Figure 2 Patients with mutations in *ZIC2*, arranged by holoprosencephaly (HPE) type. Letters link to patients described in tables 2 and 5. Note the pattern of facial findings in patients with mutations in *ZIC2*, consisting of bitemporal narrowing, upslanting palpebral fissures, flat nasal bridge, a short nose with upturned nares, a broad and/or deep philtrum, and the appearance of large ears. MIHV, middle interhemispheric variant.

Table 5 Description of common dysmorphic features in probands with photos available for review. Image references are shown in the far right column, with the corresponding identifier for table 2 and figure 2. Images were independently reviewed by three co-authors (KR, BS, MM)

Patient	HPE type	BN	USPF	FNB	SNAN	BDP	LE*	Other	Reference (for images)	figure 2	table 2
1	A	+	+	+	+	+		Synophrys	This report	a	49
2	A		+	+	+				This report	b	108
3	A	+	+	+	+	+		Tall forehead	This report	N/A	24
4	A	+	+			+			This report	N/A	27
5	A	+	+	+	+	+			17	N/A	52
6	A		+				+	Sloping forehead	17	N/A	82a
7	A		+		+		+	Sloping forehead	This report	N/A	82b
8	S		+		+				This report	c	2
9	S	+				+		Tall, narrow head, exotropia	This report	d	62
10	S	+	+		+		+		This report	e	28a
11	S		+				+	Synophrys	This report	f	102
12	S	+	+	+	+	+			This report	g	68a
13	S		+		+	+	+	Slight synophrys, cupid-bow upper lip	This report	h	22
14	S		+	+				Broad forehead	This report	i	45
15	S		+	+	+				This report	j	91
16	S		+		+	+			This report	k	26
17	S		+		+		+	Synophrys	This report	l	59
18	S		+		+		+		This report	m	99
19	S		+		+		+		This report	n	48
20	S	+	+	+	+	+	+	Tall, broad forehead	This report	o	58
21	S	+	+			+		Tall forehead, dysplastic ears	This report	N/A	64
22	S	+	+						This report	N/A	19a
23	S	+	+			+			This report	N/A	19b
24	S	+	+		+		+	Tall forehead	24	N/A	89
25	S	+	+		+	+			24	N/A	61a
26	S	+	+	+	+			Triangular mouth, myopathic facies	24	N/A	43
27	L	+	+	+	+				24	N/A	16a
28	MIHV		+		+		+		This report	p	93
29	MIHV		+		+				This report (pictured in reference[24] at younger age)	q	79
30	Unknown	+	+		+	+		Tall forehead	This report	N/A	110

*Measurements not universally available; and are the subjective judgement of independent dysmorphologists.
BDP, broad or deep philtrum; BN, bitemporal narrowing; FNB, flat nasal bridge; LE, large ears; MIHV, middle interhemispheric variant; SNAN, short nose and/or anteverted nares; USPF, upslanting palpebral fissures.

more commonly associated with HPE, such as severe hypotelorism, flat nasal bridge, cleft lip/palate, or SMCI, is striking. Although the retrospective data do not allow certain comparisons to be made, available statistical calculations show evidence that the facial phenotype in patients with *ZIC2* mutations is different than that of other cohorts of patients with HPE.

Second, unlike other genes associated with HPE, the majority of mutations in *ZIC2* occur de novo. Our data suggest the presence of at least five families in which germline mosaicism appears to be causative of HPE in a child, which has important implications for genetic counselling. 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 found to have a mutation, which is strikingly different from what has been observed with 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, and again is important for genetic counselling.

Finally, our findings show that non-chromosomal, non-syndromic HPE is not simply an 'above-the-neck' diagnosis. Patients with mutations in *ZIC2* frequently have other organ systems involved, and clinicians must look beyond craniofacial and structural brain anomalies in their clinical assessment. While skeletal anomalies may be more frequent in patients with *ZIC2* than in other types of non-chromosomal, non-syndromic HPE, no clear overall pattern emerges except that it is important to be aware that congenital anomalies may be found in other major organ systems.

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 in synthesising 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 analysis reveals a previously unnoticed *ZIC2* specific phenotype and highlights the importance of a comprehensive and collaborative approach in studying HPE and other complex genetic disorders.

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Table 6 Findings (beyond holoprosencephaly (HPE) type) in patients with mutations in *ZIC2* with sufficient data for analysis of findings versus reported findings in patients with mutations in *SIX3* (n=91 for facial findings, n=83 for other findings) and separately for a cohort of patients with non-syndromic HPE (n=258).[8 21] In this latter comparison, non-syndromic HPE includes patients in whom HPE does not occur in the context of a broader syndrome, but patients may still present with findings beyond the traditional craniofacial and structural brain anomalies most commonly recognised as classic features of autosomal dominant monogenic HPE[21]

	Intragenic *ZIC2* mutations % (n=59 for facial findings; n=64 for other features)	Intragenic *SIX3* mutations[21] % (p value)* (n=91 for facial findings; n=83 for other features)	Non-syndromic HPE[8] % (p value)* (n=258)	*ZIC2* deletions % (p value)* (n=15)
Hypotelorism	19	**44 (0.0015)**	N/A	N/A
Hypertelorism	12	**1 (0.0063)**	**2 (0.0008)**	**N/A**
Upslanting palpebral fissures	97	**10 (<0.0001)**	N/A	N/A
Flat nasal bridge	33	18 (0.0780)	N/A	N/A
Single maxillary central incisor	1	**9 (0.0399)**	N/A	N/A
Cleft lip/palate	10	**35 (0.0003)**	**30 (0.0003)**	20 (0.3814)
Hydrocephalus	12	4 (0.0705)	**31 (0.0006)**	**N/A**
Neural tube defects	4	2 (0.6705)	2 (0.3887)	N/A
Skeletal	14	**4 (0.0230)**	**5 (0.0075)**	**N/A**
Cardiac	9	2 (0.0878)	16 (0.1429)	**33 (0.0247)**
Renal	7	2 (0.2603)	4 (0.3788)	13 (0.3247)
Genital	7	5 (0.7379)	**17 (0.0175)**	**33 (0.0097)**
Gastrointestinal	4	1 (0.3493)	12 (0.0503)	**33 (0.0097)**
Pulmonary	4	0 (0.0500)	**0.4 (0.0381)**	13 (0.1889)

Statistical significance is shown in the right-most column, and statistically significant differences are shown in **bold**. Certain features were not analysed in all cohorts, so a comparison was not possible. Values with significantly different p values are shown in bold.
*Two-tailed p value by Fisher's exact test, compared to cohort of patients with mutations in ZIC2.

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