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The unfolding clinical spectrum of holoprosencephaly due to mutations in *SHH*, *ZIC2*, *SIX3* and *TGIF* genes

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Holoprosencephaly is a severe malformation of the brain characterized by abnormal formation and separation of the developing central nervous system. The prevalence is 1:250 during early embryogenesis, the live-born prevalence is 1:16000. The aetiology of HPE is extremely heterogeneous and can be teratogenic or genetic. We screened four known HPE genes in a Dutch cohort of 86 non-syndromic HPE index cases, including 53 family members. We detected 21 mutations (24.4%), 3 in *SHH*, 9 in *ZIC2* and 9 in *SIX3*. Eight mutations involved amino-acid substitutions, 7 ins/del mutations, 1 frame-shift, 3 identical poly-alanine tract expansions and 2 gene deletions. Pathogenicity of mutations was presumed based on *de novo* character, predicted non-functionality of mutated proteins, segregation of mutations with affected family-members or combinations of these features. Two mutations were reported previously. SNP array confirmed detected deletions; one spanning the *ZIC2*/*ZIC5* genes (approx. 100 kb) the other a 1.45 Mb deletion including *SIX2*/*SIX3* genes. The mutation percentage (24%) is comparable to previous reports, but we detected significantly less mutations in *SHH:* 3.5%vs10.7% (p=0.043) and significantly more in *SIX3:* 10.5%vs4.3% (p=0.018). For *TGIF1* and *ZIC2* mutation rate was conform earlier reports. About half of the mutations was *de novo*, one was a germ line mosaic. The familial mutations displayed extensive heterogeneity in clinical manifestation. Of 7 familial index patients only 2 parental carriers showed minor HPE signs, 5 were completely asymptomatic. Therefore, each novel mutation should be considered as a risk factor for clinically manifest HPE, with the caveat of reduced clinical penetrance.

Key words: Holoprosencephaly; SHH; SIX3; ZIC2; TGIF; genotype-phenotype

INTRODUCTION

Holoprosencephaly (HPE; MIM236100) is a structural anomaly of the human brain that occurs in early embryonic life. The disorder originates around the fourth week of pregnancy when the prosencephalon (forebrain) fails to cleave or incompletely cleaves into diencephalon and telencephalon, into distinct left and right hemispheres and into olfactory and optic bulb tracts. The estimated prevalence of HPE is 1 in 16000 after birth, but the prevalence of HPE in early embryogenesis is much higher (1 in 250)[1]. Classification is based on severity of the brain malformation and ranges from the most severe alobar form (single primitive ventricle, >60% of the cases), to the semi-lobar (incomplete separation of the ventricles, around 25% of the cases) and the lobar form (complete separation with continuity across the cortex)[2,3]. Another mild form of HPE, called middle inter-hemispheric variant is characterized by failure of separation of the posterior frontal and parietal lobes[4]. In many but not all cases the cerebral defects or classifications correlate very well with the facial appearance, better known as "the face predicts the brain"[5]. These facial appearances range from severe anomalies such as cyclopia, anophthalmia and proboscis to mild such as midline cleft lip/palate, hypotelorism, flat nose, microcephaly and single maxillary central incisor. Additionally, craniofacial malformations, neurological features, endocrine disorders, oromotor dysfunction and dysautonomic dysfunction can be observed[1].

The etiology of HPE is very heterogeneous and both environmental as well as genetic causes are known to be involved. Environmental factors, including maternal insulin-dependent diabetes mellitus, alcoholism and exposure to drugs or infections are the primary cause for only a very small percentage of patients[1]. Approximately 25% of patients comprise monogenic syndromes in which HPE is one of the malformations and about 40% of HPE patients harbor a chromosomal aberration[6]. Non-syndromic HPE in the remaining 35% of patients is caused by single-gene defects with reduced penetrance. Several genes have been identified to cause HPE including *SHH* [7], *SIX3* [8], *ZIC2* [9], *TGIF* [10], *GLI2* [11], *PTCH1* [12] and *DISP1* [13]. In this study, we screened the four major genes known to be involved in HPE namely *SHH*, *ZIC2*, *SIX3* and *TGIF* by sequence and MLPA analysis in a Dutch cohort of 86 sporadic and familial non-

syndromic HPE cases with a variable clinical phenotype. When a large deletion was detected, an additional SNP-array was performed, to determine the exact position and size of the deleted region.

MATERIALS AND METHODS

Patient Population

Between September 2001 and December 2008 a total of 86 index patients with a normal karyotype were referred to us for holoprosencephaly (HPE) genetic testing. These patients included 18 fetuses, 18 neonatal deceased children, 42 children and 8 adults. The department of Clinical Genetics Maastricht is the only center in the Netherlands performing genetic testing for HPE. Referral for HPE screening was based on the presence of clinical features belonging to the HPE spectrum (see Table 1). If the index patient carried a mutation, parents and family members were tested (54 in total). Informed consent was obtained from all patients (or parents) and family members, in accordance with guidelines of the local medical ethical committee. All families with a mutation were contacted and informed consent for publication of data and photos was obtained.

PCR and sequence analysis

Blood samples (in duplo), cultured fibroblasts or amniotic fluid cells were used for DNA extraction following standard procedures. Additionally, blood samples from both parents and additional family members were obtained when possible. Genetic analysis for the complete coding regions of genes *SHH* (HPE3,MIM#600725), *SIX3* (HPE2, MIM#603714), *ZIC2* (HPE5, MIM#603073) and *TGIF* (HPE4, MIM#602630) was performed by direct sequence analysis in both directions using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit and the ABI3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Primers sets, as designed by Applied Biosystems (AB) for VariantSEQr SNP discovery or custom designed primers following the same standards were used. Full details of primer sequences, amplicon lengths and PCR conditions are presented in Supplementary Table 1. Sequences were aligned with known

coding sequences (Genbank cDNA accession numbers: *SHH*; NM_000193.2, *SIX3*; NM_005413.2, *ZIC2*; NM_007129.2, *TGIF*; NM_003244.2) and mutations were confirmed on DNA extracted from the second blood sample if available. The frequency of novel mutations was determined in a control population of >100 healthy controls. Non-paternity was excluded in case of *de novo* mutations by multiplex VNTR analysis using the AmpFℓST® Identifiler PCR Amplification kit (Applied Biosystems, Foster City, CA).

Multiplex Ligation-dependant Probe Amplification (MLPA) analysis

A specific MLPA kit (SALSA MLPA kit P187, MRC-Holland bv, Amsterdam, The Netherlands) was developed to detect deletions/duplications within the coding regions of known HPE genes. This kit contains probes for all exons of genes *SHH, SIX3, ZIC2* and *TGIF*. Details of the probes used for the MLPA procedure as well as standard protocols can be found at website http://www.mlpa.com/pages/p187pag.html. In case a deletion/duplication was detected with MLPA, they were confirmed with either FISH using a region/gene specific probe and/or SNP array.

Affymetrix 250K *Nsp* I SNP array

The 250K *Nsp1* SNP genotyping array analysis (Affymetrix Inc., Santa Clara, CA) was performed according to the manufacturer's array specific protocol. Briefly, 250 ng of DNA was digested with *Nsp1*, adaptors were ligated followed by PCR amplification. Subsequently the PCR product was fragmented, labeled and hybridized to the 250K *Nsp1* SNP array. Genotype calls were obtained and analyzed using the Affymetric GTYPE analysis tool embedded in the GeneChip Operating Software (GCOS). For copy number estimation data were processed using the CNAT genotyping console (©Affymetrix). The patients mapping data were analyzed against 50 reference data sets obtained on the same platform from unaffected individuals.

RESULTS

In total, we detected 21 mutations in 86 patients (24%) (table 1, supplementary table 2). The mutations included 2 gene deletions, 2 nonsense mutations, 5 frame shift mutations, 1 in-frame deletion, 1 repeat-expansion mutation (3x), 1 splice-site mutation and 7 missense mutations. Pathogenicity of mutations was based on several features including the *de novo* character of mutations, segregation with clinical features of HPE, exclusion of being a polymorphism (absence in control/HPE population), conservation and location of mutation. A overview of all detected mutations with genetic and clinical features is depicted in tables 1 and 2. A summary of all currently known mutations in the four genes, including this study, is given in supplemental table 2. Some of our detected mutations were included in recent global studies to study number, type and location of mutations. No other information such as sex, clinical characteristics, inheritance, familial history etc was included in these studies [14-16].

The distribution of the 21 mutations over the genes was 3 in *SHH* (14%), 9 in *ZIC2* (43%), 9 in *SIX3* (43%) and 0 in *TGIF* (0%). Eleven (52%) mutations occurred *de novo* and non-paternity was excluded in all of them.

SHH mutations

We detected 3 mutations in the *SHH* gene. Mutation c.148_166del19 (p.Asn50X) was detected in an aborted male fetus (21^{+4} weeks) with (semi)lobar HPE, median cleft lip/palate, hypotelorism, an underdeveloped nose and left isomerism of the lungs. The father showed microcephaly, hypoplastic alae nasi, thin upper lip and long uvula and carried the mutation as well as the paternal grandfather. Also a second pregnancy was terminated (14^{+3} weeks) due to HPE signs detected with ultrasound (cleft lip/palate, hypotelorism, underdeveloped nose) and detection of the mutation in amniotic cells (pedigree 1/figure 2, picture A and B/figure 3). This couple underwent pre-implantation genetic diagnosis (PGD) and delivered a healthy son in August 2008. Mutation c.304 T>C (p.Cys102Arg) was detected in an aborted female fetus (23 weeks) with alobar HPE and arose *de novo*. The autopsy report listed facial dysmorphism including hypotelorism and a flat nose. Mutation c.1145 T>C (p.Leu382Pro) was detected in a 46-year-old female showing no HPE clinical characteristics. She was tested because her first child

died of HPE at the age of 6 months. This child was diagnosed with alobar HPE, cleft palate and epilepsy but no DNA was available for testing. The mother's asymptomatic father also carried the mutation (pedigree 3/figure 2).

ZIC2 mutations

We detected 9 mutations in the *ZIC2* gene. Mutation c.479delC (p.Pro159ArgfsX58) was detected an 8-year-old boy with lobar HPE, deafness, mental retardation and spastic diplegia. Both parents were healthy and non-carriers but one older brother died of alobar HPE three weeks after birth. Cultured fibroblasts of this deceased older brother showed that he was carrier of the same mutation (pedigree 4/figure 2) suggesting germ line mosaicism. The mutation was not detected in semen of the father. Marker analysis indicated that the risk haplotype, shared by the two affected brothers and not by a healthy brother, was inherited from the mother (data not shown). Mutation c.1090 C>T (p.Gln364X) occurred *de novo* and was detected in a female first child with semilobar HPE including microcephaly, hypotelorism and a very slow general development (picture C1-C3/figure 3). Mutation c.1325delC (p.S442SfsX112) occurred *de novo* and was detected in an aborted female fetus with lobar HPE and no further facial anomalies. The previously described alanine tract expansion mutation c.1377_1406dup30 (p.A461_470dup) was detected in three unrelated families: In the first family (7430) the mutation was detected in a male child with semilobar HPE, microcephaly, mental retardation, diabetes insipidus and tetraparesis. The boy does not speak and does not walk at currently age 8. The asymptomatic mother with a normal brain MRI carried the expansion (pedigree 7/figure 2, picture D, E, F/figure 3). In the second family (12940) the alanine tract occurred *de novo* and was detected in an aborted male fetus (23^{+4} weeks) with semilobar HPE (picture G/figure 3) and no apparent facial dysmorphism except for a broad mouth. In the third family (14475) the expansion also occurred *de novo* and was detected in an aborted female fetus (30^{+6} weeks) with semilobar HPE without further external malformations (picture H/figure 3). Mutation c.1448 G>A (p.G483D) was found in an aborted female fetus (14 weeks) with alobar HPE in combination with an encephalocele (pedigree 10/figure 2). The mother also carried the mutation but further clinical examination of the mother showed no microsigns of HPE. She refused an MRI or further familial genetic

screening. Mutation c.1508_1520del13 (p.G503AfsX48) occurred *de novo* in a 1-year old boy with semilobar HPE (picture I/figure 3) hypotelorism and a very slow mental development. The *ZIC2* deletion detected with MLPA analysis was found in an elderly female with mental retardation (picture J, figure 3). The deletion was confirmed with FISH analysis and the 250k *nsp* I Affymetrix SNP array. The deletion was too small to be detected by the default algorithms but with the exact location known could be determined at approximately 100 kb in size including the *ZIC2* and *ZIC5* genes. DNA of parents was not available.

SIX3 mutations

We detected 9 mutations in the *SIX3* gene. Mutation c.235 A>G (p.M79V) occurred *de novo* and was detected in an aborted female fetus (18^{+3} weeks) with alobar HPE and microcephaly, a single nostril, flat face and a malformed upper lip without cleft. *De novo* mutation c.404_407dupGCGC (p.V137RfsX18) was detected in a 10-month-old girl with semilobar HPE (partial hypoplasia of the corpus callosum)(picture K/figure 3). Mutation c.463_465delCAC (p.H155del) was found in a baby girl who died shortly after birth due to HPE, bilateral cleft lip and palate and a single nostril. The mutation was also detected in the asymptomatic mother (pedigree 15/figure 2). Mutation c.736delA (p.T245fsX5) occurred *de novo* in a baby girl that died 12 days after birth because of alobar HPE with a single nostril, flat nose, median cleft lip/palate and hypotelorism. Mutation c.769 C>G (p.R257G) was detected in a 16-year-old boy with lobar HPE, microcephaly and mental retardation. A younger sister died the second day after birth. The autopsy report on the sister did not indicate specific HPE characteristics as the forebrain was completely divided and the corpus callosum was present. There were however brain abnormalities including brain atrophy, absent nervi olphactorii and large ventricles. DNA of the sister was not available. The asymptomatic father carried the mutation, but his parents did not (pedigree 17/figure 2). Mutation c.806 G>T (p.R269M) was detected in a 21-year-old man (picture L/figure 3, photo taken at teenage years) with semilobar HPE cleft lip/palate, diabetes insipidus, epileptic fits and severe mental retardation (pedigree 18/figure 2). Clinical diagnosis of HPE was made at age 4[17]. Two younger children of this family died shortly after birth due to HPE but DNA of them was not available[17]. The mother has a solitary

median maxillary incisor (SMMCI) and carries the mutation (picture M/figure 3). Mutation c.806 G>C (p.R269T) was detected in an asymptomatic 60-year-old male who was screened because of multiple HPE related deaths in his family (II-5, pedigree 19/figure 2). A sister (II-2, bilateral cleft lip/palate, microcephaly), brother (II-3, cervical rachischisis, missing vertebrae), a niece (III-2, HPE, midface hypoplasia, high palatum), a nephew (IV-1, HPE, cleft lip/palate, absent nose, hypotelorism, picture N/figure 3) and two daughters (III-4, III-5, HPE, bilateral cleft lip/palate, hydrocephalus) showed characteristics within the HPE spectrum. DNA was not available for testing. The nephew (IV-1, picture N/figure3) and asymptomatic father (III-1, picture O/figure3)of the index both carried the mutation (pedigree 19/figure 2). Mutation c.806+1 G>T occurred *de novo* in an 8 month-old boy with semilobar HPE, microcephaly, epilepsy, psychomotor retardation and diabetes insipidus who died at age 2. The *SIX3* gene deletion detected with MLPA occurred *de novo* in an aborted male fetus (23^{+4} weeks) with semilobar HPE and no facial dysmorphism (picture P/figure 3). The deletion was confirmed with FISH analysis and the *nsp* I 250k Affymetrix SNP array. The deletion was approximately 1,45 Mb in size (Nucleotide positions 43845329 to 45152135, 194 SNP's, hg. 19/NCBI Build 37), including the *SIX3* and *SIX2* genes at the centromeric end of the deletion (figure 1).

DISCUSSION

Screening the four major HPE genes (*SHH*, *SIX3*, *ZIC2* and *TGIF*) in 86 Dutch index patients and 54 family members led to the detection of 21 heterozygous mutations (24.4%). This mutation rate is comparable to previous reports for non-syndromic HPE [18]. Only the alanine tract expansion in the *ZIC2* gene and the c.404_407dupGCGC mutation in the *SIX3* gene have been identified previously [9,19,20]. Of the 31 family members tested for a familial mutation, 9 were carriers of whom 4 showed major or minor HPE signs. Therefore around 55% of mutation carriers were completely asymptomatic.

We detected only 3/86 mutations in *SHH* (3.5%), which is significantly lower than the previously reported 121/1100 (10.7%) by Roessler et al. [15] ($p=0.047$, $\chi^2=3.94$). Two of the mutations were familial and showed extremely wide phenotypic variability within the family as previously described[18]. The

p.N50X mutation truncates the SHH protein in the SHH-N domain, thereby eliminating the Hint motif necessary for autocatalytic processing and is considered a biologically null allele[15]. Missense mutation p.C102R is also located in the important SHH-N region of the protein and is very highly conserved in other species up to *c. elegans*. Combined with the *de novo* character of this mutation, pathogenicity of this mutation is a likely explanation for the alobar HPE. Missense mutation p.L382P is located in the SRR region, in which several HPE mutations have been detected and which functions to bind cholesterol[15].

We detected 9/86 mutations in *ZIC2* (10.5%), a percentage comparable to a recent summarizing study from Roessler et al.[16]. Consistent with previous studies [9,16,21] we found: 1) a high frequency (>55%) of frame shifts, nonsense mutations and gene deletions (Table 1), 2) >50% *de novo* mutations, 3) several frame shift mutations in the C-terminal end of the ZIC2 protein, leaving the zinc-finger domains theoretically intact, 4) the poly-alanine tract expansion as a prevalent mutation type. Inconsistencies with previous studies were also found: in two of three patients the alanine tract expansion was *de novo*, in the third patient maternally inherited, therefore not predominantly from the father as previously described. Additionally, the mother was not a mosaic carrier of the mutation[19]. The one missense mutation we detected, p.G483D, is located in the C-terminal end of the ZIC2 protein where so far only frame shifts and ins/del mutations have been detected. This mutation does not occur in a conserved region and the glycine amino-acid is not well conserved among species. However, as the mutation was not observed in a panel of 110 healthy controls and was never described in literature (>1000 HPE index patients) it is very unlikely to be a common polymorphism. The aborted fetus showed alobar HPE without facial anomalies and the asymptomatic mother also carried the mutation. Since minor/absent facial dysmorphism combined with severe brain malformations and incomplete clinical penetrance are specifically consistent with *ZIC2* mutations, we consider this variant most likely pathogenic.

Considering all 21 index patients with a mutation (table 1 and 2) shows that facial malformations in the *ZIC2* patients are less apparent than in patients with mutations in the other genes. This milder facial phenotype with usually no clefts or single nostrils as seen in genes as *SIX3* and *SHH* is consistent with literature [1,19,22]. The specific facial features (bitemporal narrowing, upslanting palpebral fissures, flat nasal

bridge, short nose, broad philtrum and large ears) as described recently by Solomon et al.[22] could in part be fitted to our patients; specifically patient C, D and I fit several of these features. Distribution of HPE classifications (lobar, semilobar, alobar) were however mixed and not different from patients with other mutated genes. We detected 9/86 mutations in *SIX3* (10.5%) a percentage significantly higher than published by Lacbawan et al. [14] who detected 34/800 (4.25%) mutations in *SIX3* (p=0.018, χ^2=5.63). The more prevalent maternal inheritance and a 1.5:1 female : male ratio described in this study could not be confirmed in our population (table 1), probably due to the smaller sample size. Our population however does seem to support the more severe phenotype in *SIX3* index patients (table 2) [14,23] and a high penetrance of clinical symptoms within familial mutation carriers. In a recent study Domené et al. [20] developed a set of robust and sensitive assays in zebra fish to functionally test *SIX3* mutations and characterized 46 mutations. The already known *SIX3* mutation c.404_407dupGCGC (table 1) was also characterized in this study and yielded <50% of activity in the overexpression assay (c.q. ability to induce dorsalised phenotypes), but retained activity in the rescue assay (c.q. ability to rescue the *headless* phenotype) [20]. Interestingly they characterized two different mutants in codon 257, namely c.769 C>T (p.R257W) and c.770 G>C (p.R257P) leading to different amino acid substitutions and completely opposite functional properties. As we detected yet another mutation in this codon c.769 C>G (p.R257G), and this codon therefore appears to be prone to mutation, it would be interesting to see what phenotype this mutation gives in the assay. A second nearby codon susceptible to mutation is amino acid p.R269. Domené et al. characterized c.807 G>C (p.R269S) which retained intermediate activity (50-90%). We detected two other mutations c.806 G>T (p.R269M) and c.806 G>C (p.R269T) in the same codon and a splice mutation c.806+1 G>T. These mutations would also be very interesting in functional assays and would maybe explain why these two arginine positions at the end of Homeo-domain and C-terminal motifs are so apparently prone to mutate.

We did not detect any mutation in the *TGIF* gene. This is not statistically different from other studies and our population may have been simply too small as percentage of *TGIF* mutations is very low (1-2%). The added value of a technique to determine duplications or deletions such as MLPA for the 4 major HPE

genes is evident as we detected two gene deletions, representing 12% of the total number of mutations detected. Earlier, Bendavid et al.[24] already described several microdeletions in other genes (*SHH* and *TGIF*) using the subtelomeric MLPA kit as well as precisely sized duplications and deletions at HPE loci using array-CGH[25]. It is therefore clear that complete gene deletions of all major HPE genes are possible and should be included in diagnostic testing.

Our future research will focus on increasing the mutation rate (currently 24%), as not all currently known HPE genes are as yet routinely tested and a large portion of the non-syndromic patients with a normal karyotype remain unexplained. We aim at including *GLI2* (MIM#165230, HPE9), *PTCH1* (MIM#601309, HPE7) and *TDGF1* (MIM#187395) in the near future. Furthermore, recent studies [24,25] have indicated more candidate loci for HPE, which may also increase the current mutation rate. Obviously, functional *in vitro/in vivo* studies that may elucidate biological and molecular pathways and/or discriminate between the functional effects of unique mutations are imperative in further unraveling the HPE etiology. A major future challenge will be the search for additional genetic or environmental risk factors that modulate or even evoke the phenotypic variability within families.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTAL DATA

Supplementary information is available at The European Journal of Human Genetics' website.

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Table 1: Mutations detected in the Dutch population

Nr	Gene	Fam nr.	Mutation	Amino-acid change	Mode of inheritance	Age at testing (Sex)	Picture (fig 3)
1	*SHH*	10304	c.148_166del19	p.N50X	Paternal	fetus (M)	A/B
2		10959	c.304 T>C	p.C102R	*De novo*	fetus (F)	
3		14317	c.1145 T>C	p.L382P	Paternal	46 (F)	
4	*ZIC2*	8774	c.479delC	p.P159RfsX58	Germline	8 (M)	
5		9004	c.1090 C>T	p.Q364X	*De novo*	0 (F)	C
6		14914	c.1325delC	p.S442SfsX112	*De novo*	fetus (F)	
7		7430	c.1377_1406dup30	p.A461_470dup	Maternal	1 (M)	D/E/F
8		12940	c.1377_1406dup30	p.A461_470dup	*De novo*	fetus (M)	G
9		14475	c.1377_1406dup30	p.A461_470dup	*De novo*	fetus (F)	H
10		14919	c.1448 G>A	p.G483D	Maternal	fetus (F)	
11		11643	c.1508_1520del13	p.G503AfsX48	*De novo*	1 (M)	I
12		9723	Gene deletion	-	unknown	62 (F)	J
13	*SIX3*	12248	c.235 A>G	p.M79V	*De novo*	fetus (F)	
14		9823	c.404_407dupGCGC	p.V137RfsX18	*De novo*	1 (F)	K
15		10482	c.463_465delCAC	p.H155del	Maternal	$0^{\dagger}$ (F)	
16		8616	c.736delA	p.T245fsX5	*De novo*	$0^{\dagger}$ (F)	
17		8449	c.769 C>G	p.R257G	Paternal	16 (M)	
18		7583	c.806 G>T	p.R269M	Maternal	21 (M)	L/M
19		10536	c.806 G>C	p.R269T	Paternal	60 (M)	N/O
20		5726	c.806 +1 G>T	Frameshift	*De novo*/germline	$2^{\dagger}$ (M)	
21		12519	Gene deletion	-	*De novo*	fetus (M)	P

Table 2: Typical HPE characteristics of index patients

Nr	Gene	Fam nr.	Mutation	Type of HPE	Microcephaly	Cleft lip/palate	Hypotelorism	Single nostril	Flat nose/face	Mental retardation
1	*SHH*	10304	p.N50X	(semi)lobar	X	X	X		X	
2		10959	p.C102R	alobar	X		X		X	
3		14317	p.L382P	None						
4	*ZIC2*	8774	p.P159RfsX58	lobar						X
5		9004	p.Q364X	semilobar	X		X			
6		14914	p.S442SfsX112	Lobar						
7		7430	p.A461_470dup	semilobar	X					X
8		12940	p.A461_470dup	semilobar						
9		14475	p.A461_470dup	semilobar						
10		14919	p.G483D	alobar						
11		11643	p.G503AfsX48	semilobar			X			X
12		9723	gene deletion	semilobar						X
13	*SIX3*	12248	p.M79V	alobar	X			X	X	
14		9823	p.V137RfsX18	semilobar						
15		10482	p.H155del	n.s.		X		X		
16		8616	p.T245fsX5	alobar		X	X	X	X	
17		8449	p.R257G	lobar	X					X
18		7583	p.R269M	semilobar		X				X
19		10536	p.R269T	none. obligate carrier (son)	(X)	(X)	(X)	(X)	(X)	
20		5726	c.806 +1 G>T (fs)	semilobar	X					X
21		12519	gene deletion	semilobar						

n.s. = not specified, in brackets () clinical characteristics of son of asymptomatic obligate carrier

TITLES AND LEGENDS TO FIGURES

Figure 1: Characterization of SIX3 gene deletion

Family 12519 with the *SIX2/3* deletion originally detected with MLPA and confirmed with by 250K Affymetrix SNP array (194 SNP's =>1.45 Mb). The deleted area is highlighted and zoomed in with the *SIX2/3* genes at the centromeric end of the deletion.

Figure 2: Pedigrees of familial mutations

Family pedigrees according to numbering of Tables 1 and 2 are indicated at the top left corner of each pedigree. Specific mutation for the family is indicated in the right upper corner. + indicates mutation carriers; - indicates non-mutation carriers; squares indicates males, circles females; filled symbols are patients with HPE clinical characteristics; arrows indicate family index.

Figure 3: Pictures of patients

Pictures of HPE patients or their relatives. Letters below the pictures are referred in the text as well as in table 1. All photographs are published with informed consent of index patients or their parents.

Supplemental Table 1: PCR primers for *SHH*, *ZIC2*, *SIX3* and *TGIF* genes

Gene	Exon	Primer sequence 5'→3'	Amplicon length	cDNA length	PCR mix
SHH	1	F: TGTAAAACGACGGCCAGTGGGAAGGAGCGGGTGAAATC R: CAGGAAACAGCTATGACCGAGAGCGCACACGCACACAC	514	451	VS standard
	2	F: TGTAAAACGACGGCCAGTGGCACCTCACAGGGCAGGTT R: CAGGAAACAGCTATGACCGGTGTGTCTGGGCCATTCCA	577	262	VS standard
	3A	F: TGTAAAACGACGGCCAGTGTGCGGCGCCACAAAGAG R: CAGGAAACAGCTATGACCCCAGCCAACCTCTGTGACCG	526	269	VS standard
	3B#	F: TGTAAAACGACGGCCAGTACGGCGCCAAGAAGGTCTT R: CAGGAAACAGCTATGACCCCCAGCTGTGCTCCTCGAT	381	381	VS[1]
	3C#	F: TGTAAAACGACGGCCAGTGCCCGGGCCAGCGCGTGTACGTGG	313	313	VS[2]
	3D	F: GCAGAGTAGCCCTAACCG R: CATACCAAACAAATAAATAGCCAG	438	195	Invitrogen
ZIC2	1A	F: CTGGAGCCCGGTGGCCGCC R: CGGCGTGGGGCCCGAGCG	407	307	VS[3]
	1B#	F: TGTAAAACGACGGCCAGTCGCACATGGGAGCCTTCAA R: CAGGAAACAGCTATGACCCGGCTGCTGCCATGTTCA	522	522	VS standard
	1C#	F: TGTAAAACGACGGCCAGTGGCGCAACTCCACAACCAGT R: CAGGAAACAGCTATGACCAGTCTCAGCCTGTGCGTGTCTC	514	455	VS standard
	2#	F: TGTAAAACGACGGCCAGTCAGAGGGGATCGGACTGGTT R: CAGGAAACAGCTATGACCAGCACCCCCAGAAATGTTCC	436	164	VS standard
	3	F: GGGGAACATTTCTGGGGGTG R: ATCACAAGGTGCCCTCGCTGCT	547	444	Invitrogen
SIX3	1A	F: GGTGTGGGTGTCCCTTACGCC R: GCAGCTGGAACATGGACAACTCTT	424	424	Invitrogen
	1B	F: ACCACCGCTCCATACTTCTG R: TGTGGTTCTCAAGGATGTGG	399	399	Invitrogen
	1C#	F: TGTAAAACGACGGCCAGTCTTCCACACGGGCAACTTCC R: CAGGAAACAGCTATGACCCTCCGACTGGCCAGGAGTCA	514	390	VS standard
	2	F: CGTACTTTGTCGCTTGCACC R: CGGTTTGTTCTGGGGATGG	497	311	Invitrogen
TGIF	2	F: TGTAAAACGACGGCCAGTACTCTGACAGCGCCGAGGTG R: CAGGAAACAGCTATGACCGGCGCTGAGCGTGTTTCTTG	588	184	VS standard
	3	F: TGTAAAACGACGGCCAGTGGCATTTGGTTGAGAGCCTCC R: CAGGAAACAGCTATGACCTGACTTGACACACAGGAATGCC	510	227	VS standard
	4A	F: TGTAAAACGACGGCCAGTGCTTGCTTTCTTGGCGGAGC R: CAGGAAACAGCTATGACCTGGGTACATGAGAGAGGTGTCT	581	405	VS standard
	4B	F: TCTCAGAACCCGTTGGCT R: TCATCTCTTGCCTTCACC	700	639	VS standard

in house design, underlined: M13-sequence

Supplemental Table 2: Known HPE mutations in *SHH, SIX3, ZIC2* and *TGIF* genes

SHH mutations

Mutation	Amino-acid change	Literature
c.9_12dup	p.R6GfsX59	[26]
c.17 G>C	p.R6T	[21]
c.38_45del	p.V13AfsX48	[26]
c.50 T>C	p.L17P	[27]
c.72 C>A	p.C24X	[21]
c.77 C>T	p.P26L	[28]
c.80 G>C	p.G27A	[29]
c.87delG	p.G29fsX11	[15]
c.91 G>A	p.G31R	[7,30]
c.108_109delinsG	p.K38SfsX2	[15]
c.116 T>C	p.L39P	[15]
c.119_120delCC	p.P41FfsX22	[15]
c.136 C>T	p.Q46X	[15]
c.148_166del19	**p.N50X**	**This study,** [15]
c.157 G>A	p.E53L	[15]
c.210dupC	p.E71RfsX4	[15]
c.211delG	p.E71SfsX27	[21]
c.248 A>T	p.D83V	[15]
c.250 A>T	p.I84F	[15]
c.263 A>T	p.D88V	[26]
c.298delC	p.Q100RfsX7	[15]
c.298 C>T	p.Q100X	[7]
c.300 G>C	p.Q100H	[21,31,32]
c.304 T>C	**p.C102R**	**This study,** [15]
c.305 G>A	p.C102Y	[15]
c.313 A>T	p.K105X	[7,30]
c.316_321del	p.L106_N107del	[21]
c.327 G>T	p.L109F	[15]
c.328 G>A	p.A110T	[15]
c.329 C>A	p.A110D	[21]
c.331 A>T	p.I111F	[33]
c.332 T>A	p.I111N	[34]
c.345 C>A	p.N115K	[26]
c.349 T>G	p.W117G	[7]
c.349 T>C	p.W117R	[7]
c.355 G>T	p.G119X	[15]
c.370 G>A	p.V124M	[15]
c.383 G>A	p.W128X	[35]
c.388 G>T	p.E130X	[21]
c.406 G>A	p.E136K	[32]
c.415_416del2	p.L139AfsX13	[15]
c.419 A>C	p.H140P	[36]
c.420 C>G	p.H140Q	[37]
c.428 G>A	p.G143D	[15]
c.431 G>C	p.R144P	[15]
c.439 G>A	p.D147N	[15]
c.449 C>G	p.T150R	[21]
c.449 C>A	p.T150K	[15]
c.468 C>A	p.S156R	[15]
c.474 C>G	p.Y158X	[31]
c.509 T>G	p.F170C	[15]

c.511 G>C	p.D171H	[15]
c.525 C>G	p.Y175X	[15]
c.527_535del9	p.E176_K178del	[21]
c.547 T>C	p.C183R	[15]
c.548 G>T	p.C183F	[36]
c.548 G>A	p.C183Y	[15]
c.551 C>T	p.S184L	[15]
c.562 G>C	p.E188Q	[21,31]
c.562+1 G>A	splicing	[15]
c.584_598del16	p.G196_200del	[15]
c.587delG	p.G196EfsX20	[15]
c.587 G>A	p.G196E	[15]
c.590 G>T	p.G197V	[15]
c.592 T>A	p.C198S	[15]
c.593 G>A	p.C198F	[32]
c.624_625delinsTT	p.E208DfsX1	[15]
c.625 C>T	p.Q209X	[26]
c.653 T>C	p.L218P	[37,38]
c.664 G>A	p.D222N	[21,31]
c.671 T>A	p.V224E	[39]
c.676 G>A	p.A226T	[39]
c.692 G>T	p.G231V	[15]
c.694 C>G	p.R232G	[15]
c.701 T>C	p.L234P	[15]
c.707 G>A	p.S236N	[15]
c.708 C>A	p.S236R	[26]
c.721 T>C	p.F241V	[15]
c.723 C>G	p.F241L	[15]
c.764 T>A	p.I255N	[15]
c.766 G>T	p.E256X	[26,39]
c.787_807del21	p.R263_A269del	[26]
c.800 C>T	p.T267I	[29]
c.812 T>C	p.L271P	[21]
c.824 C>A	p.A275E	[15]
c.839 C>G	p.S280W	[15]
c.839 C>A	p.S280X	[37,38]
c.850 G>T	p.E284X	[29,39]
c.869 G>A	p.G290D	[26]
c.887 G>C	p.G296A	[15]
c.928 C>T	p.R310C	[15]
c.961 C>A	p.R321S	[15]
c.995 T>C	p.V332A	[21]
c.1015 G>T	p.E339X	[15]
c.1037 C>T	p.A346V	[15]
c.1040 C>G	p.P347R	[15]
c.1040 C>A	p.P347Q	[21]
c.1040 C>T	p.P347L	[15]
c.1051 C>T	p.Q351X	[15]
c.1061 T>C	p.I354T	[21]
c.1085 C>T	p.S362L	[15]
c.1088 G>A	p.C363Y	[37,38]
c.1091 A>G	p.Y364C	[15]
c.1117 G>A	p.A373T	[29]
c.1121 A>G	p.H374R	[15]
c.1127 C>A	p.A376D	[15]

c.1130 T>C	p.F337S	[15]
c.1132_1140del9	p.A378_F380del	[26]
c.1142 G>C	p.R381P	[21]
c.1145 T>C	**p.L382P**	**This study,** [15]
c.1147 G>A	p.A383T	[39]
c.1171 G>A	p.A391T	[15]
c.1204_1227del24	p.402_409del	[40]
c.1213_1227del	p.G405_G409del	[26]
c.1231_1233dup	p.G411dup	[15]
c.1246 A>G	p.T416A	[15]
c.1270 C>G	p.P424A	[26]
c.1303 T>A	p.Y435N	[15]
c.1307 C>T	p.S436L	[26]
c.1366 G>C	p.G456R	[15]
c.1370delT	p.M457RfsX18	[15]

SIX3 mutations

Mutation	Amino-acid change	Literature
c.109 G>T	p.G37C	[20]
c.206 G>A and c.406_407dupGC	p.G69D and frameshift	[23]
c.214 G>C	p.A72P	[23]
c.235 A>G	**p.M79V**	**This study,** [14]
c.275 T>G	p.V92G	[21,41]
c.278 C>A	p.A93D	[20]
c.311 A>G	p.D104G	[23]
c.313 A>G	p.I105V	[21]
c.338 G>A	p.W113X	[14]
c.339 G>C or T	p.W113C	[20]
c.341 C>T	p.S114L	[20]
c.385 G>T	p.E129X	[20]
c.389 C>A	p.S130X	[20]
c.404 C>G	p.R135P	[23]
c.404_407dupGCGC	**frameshift**	**This study,** [20]
c.405_409dupCGCCG	frameshift	[20]
c.406_407dupGC and c.206 G>A	p.G69D and frameshift	[23]
c.413 T>A	p.V138D	[14]
c.463_465delCAC	**p.His155del**	**This study,** [14]
c.469 T>A	p.F157I	[20]
c.507delG	frameshift	[23]
c.515 C>T	p.A172V	[20]
c.518 A>C	p.H173P	[21]
c.520 T>C	p.Y174H	[20]
c.542_576dup	frameshift	[21]
c.551delC	frameshift	[20]
c.556_557dup	frameshift	[21]
c.557_558insGG	frameshift	[42]
c.582dupC	frameshift	[20]
c.605 C>T	p.T202I	[21]
c.619 G>T	p.E207X	[20]
c.637 T>G	p.F213V	[20]
c.652 C>T	p.R218W	[20]
c.653 G>C	p.R218P	[20]
c.676 C>G	p.L226V	[8]
c.680 A>C	p.Q227P	[20]
c.686 C>T	p.P229L	[43]

c.692 C>G	p.P231R	[21]
c.694_702del9	p.232_234del	[8]
c.718 G>A; c.719 C>A	p.A240K	[20]
c.721 C>T	p.Q241X	[20]
c.730 G>T	p.G244C	[20]
c.736delA	**frameshift**	**This study,** [14]
c.743_745delCAG	p.248K delQ	[14]
c.749 T>C	p.V250A	[8]
c.762 T>A	p.F254L	[20]
c.769 C>T	p.R257W	[21]
c.769 C>G	**p.R257G**	**This study,** [14]
c.770 G>C	p.R257P	[8]
c.773 G>T	p.R258L	[20]
c.785 G>A	p.R262H	[20]
c.806 G>T	**p.R269M**	**This study,** [14]
c.806 G>C	**p.R269T**	**This study,** [14]
c.806+1 G>T	**Alternative splicing**	**This study,** [14]
c.807 G>C	p.R269S	[20]
c.820_832delins8	p.A274X	[14]
c.850 G>C	p.A284P	[14]
c.890 C>T	p.P297L	[20]
c.944 C>T	p.T315I	[23]
c.947 C>T	p.P297L	[23]
Del SIX3	**Gene deletion**	**This study**

ZIC2 mutations

Mutation	Amino-acid change	Literature
c.21delG	p.Q8SfsX33	[19]
c.81_86delGGCGGCinsTCGGT	p.A28RfsX13	[16]
c.107 A>C	p.Q36P	[21]
c.109 G>A	p.D37N	[16]
c.129_184dup56	p.L62Rfs175	[16]
c.136 C>T	p.Q46X	[21]
c.172 G>T	p.G58X	[21]
c.177ins56	p.F60QfsX176	[9]
c.191dupC	p.A66RfsX301	[16]
c.217 C>T	p.Q73X	[16]
c.217delC	p.Q73RfsX145	[16]
c.367delA	p.S123AfsX95	[16]
c.382 G>A	p.D128N	[16]
c.386_392delCGGCGCC	p.S129WfsX87	[16]
c.392_398del7	p.G133SfsX83	[16]
c.457_458 GA>TT	p.D153F	[9,21]
c.479delC	**p.P160RfsX58**	**This study,**[16,22]
c.490 G>T	p.E164X	[16]
c.557_572dup16	p.E192GfsX180	[16]
c.577delC	p.Q193NfsX25	[16]
c.582 C>A	p.Y194X	[16]
c.612delC	p.Y205TfsX13	[16]
c.622 GC>TT	p.A208L	[22]
c.659delA	p.N220TfsX4	[16]
c.665_676dup12	p.G222_M225dup	[16]

c.748 C>T	p.Q250X	[16]
c.779 G>A	p.W260X	[16]
c.793 C>T	p.Q265X	[16]
c.797_801del5	p.L266QfsX99	[22]
c.808_824ins17	p.K270TfsX2	[21]
c.815 G>A + c.974 G>T	p.S272N +p.R325L	[16]
c.825_826delAA	p.K275NfsX91	[16]
c.829_830dupTT	p.T279AfsX7	[16]
c.856 C>T	p.H286Y	[16]
c.857 A>T	p.H286L	[16]
c.858 C>G	p.H286Q	[16]
c.862_863delTC	p.S288GfsX78	[16,36]
c.871 C>T	p.H291Y	[16]
c.910 T>A	p.W304R	[16]
c.912 G>A	p.W304X	[16]
c.928 G>T	p.E310X	[16]
c.932delG	p.G311AfsX102	[19]
c.941 T>G	p.F314C	[16]
c.973 C>A	p.R325S	[16]
c.979 C>T	p.H327Y	[16]
c.994_1005dup	p.C335_338dup	[22]
c.1004 G>T	p.C335F	[16]
c.1025_1026delAA	p.K342SfsX24	[21]
c.1031_1032delTC	p.F344XfsX22	[19]
c.1040_1046del7	p.E348SfsX63	[19]
c.1051 A>T	p.K351X	[16]
c.1052_73ins24	p.K351_358ins	[16]
c.1075+1 T>A	p.G359fsX62	[16]
c.1076-1 G>A	Alternative splicing	[16]
c.1090 C>T	**p.Q364X**	**This study,** [16,22]
c.1091_1092delAG	p.Q364LfsX2	[19,21]
c.1095_1096delTG	p.C365X	[16]
c.1097_1098delAG	p.E366VfsX2	[19]
c.1118 G>C	p.R373P	[16]
c.1119_1120delCT	p.F374RfsX17	[16]
c.1204 T>A	p.Y402N	[16]
c.1206 C>G	p.Y402X	[16]
c.1208 C>A	p.T403K	[16]
c.1211 A>G	p.H404R	[16]
c.1225 C>T	p.R409W	[16]
c.1239+1 G>C	Alternative splicing	[16]
c.1240-2 A>G	Alternative splicing	[16]
c.1245 T>G	p.H415Q	[16]
c.1277delC	p.P426RfsX129	[16]
c.1313dupC	p.L440AfsX90	[9]
c.1323dupG	p.S442VfsX88	[19,44]
c.1325delC	**p.S442SfsX112**	**This study**
c.1329delC	p.S444AfsX111	[22]
c.1330_1365del	p.S444_455del	[19,44]
c.1366_1395dup30	p.A456_465dup	[22]
c.1377_1406dup30	**p.A461_470dup**	[9,15,19], **this study**
c.1377_1406del30	p.A456_465del	[16]
c.1392_1406del	p.A461_465del	[16]
c.1401_1406dup	p.A469_470dup	[22]
c.1420_1427dup; c.1428_1433delinsCG	p.G477CfsX54	[16]

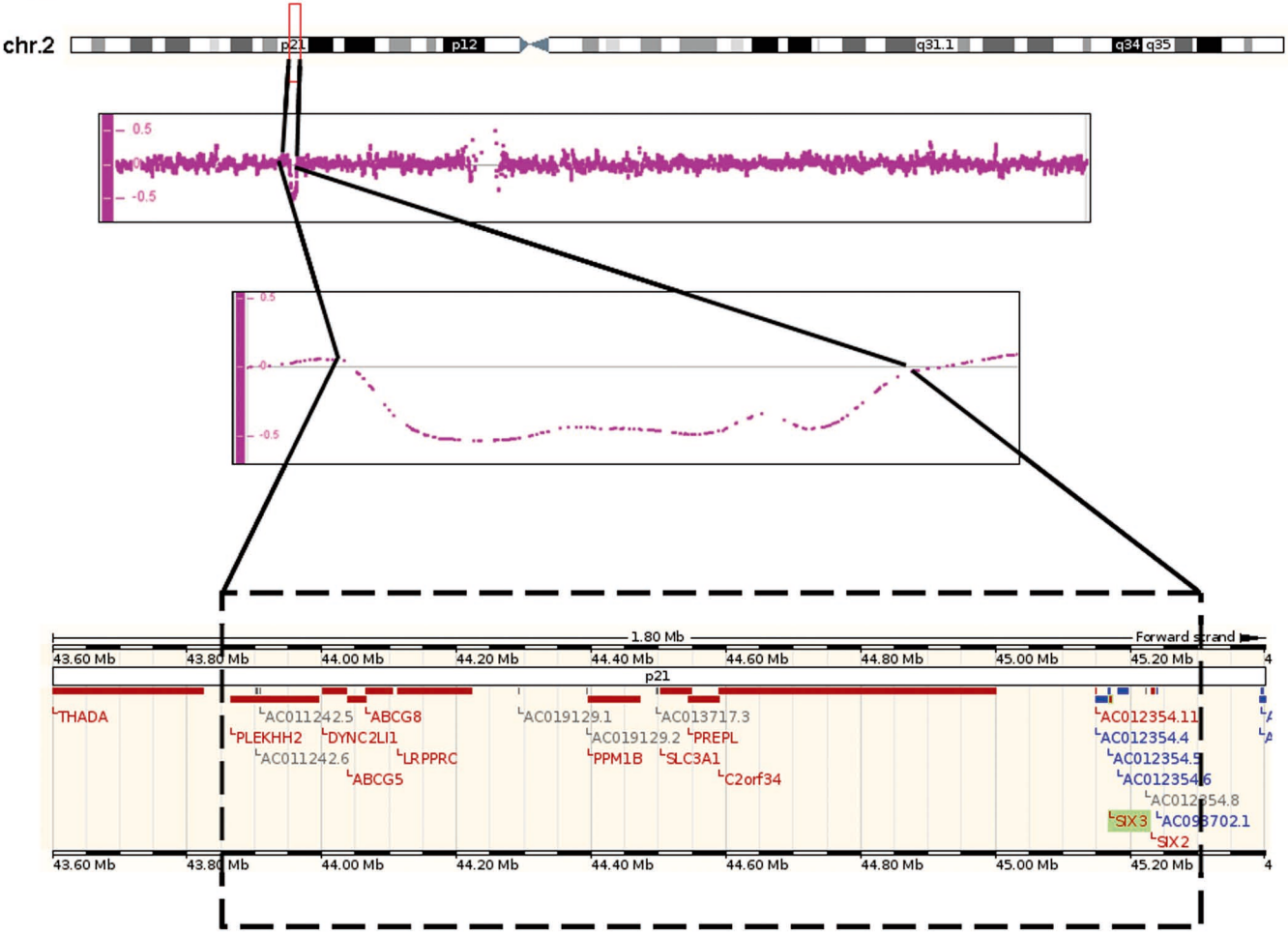
c.1437_1441del	p.S480QfsX48	[22]
c.1445_1461del17	p.S482RfsX42	[16]
c.1448 G>A	**p.G483D**	**This study**
c.1452_1456del5	p.A485RfsX43	[16]
c.1455_1461delinsCG	p.G487LfsX41	[16]
c.1520del13	**p.G503AfsX48**	**This study,** [16]
c.1559delA	p.H520PfsX35	[16]
Del ZIC2	**Gene deletion**	**This study**, [22]

TGIF mutations

Mutation	Amino-acid change	Literature
c.83 C>G	p.S28C	[10]
c.132 G>T	p.K44N	[45]
c.133 G>T	p.E45X	[46]
c.140_141delTG	frameshift	[46]
c.177 C>G	p.Y59X	[21]
c.187 C>G	p.P63A	[10]
c.228 C>A	p.H76Q	[46]
c.268 C>T	p.R90C	[47]
c.320 A>T	p.Q107L	[21]
c.377 T>C	p.V126A	[48]
c.451 A>G	p.T151A	[10]
c.485 C>T	p.S162F	[10]
c.778delC	frameshift	[46]

SHH Genbank accession NM_000193.2
SIX3 Genbank accession NM_005413.3
ZIC2 Genbank accession NM_007129.2
TGIF1 Genbank accession NM_003244.2

Figure 1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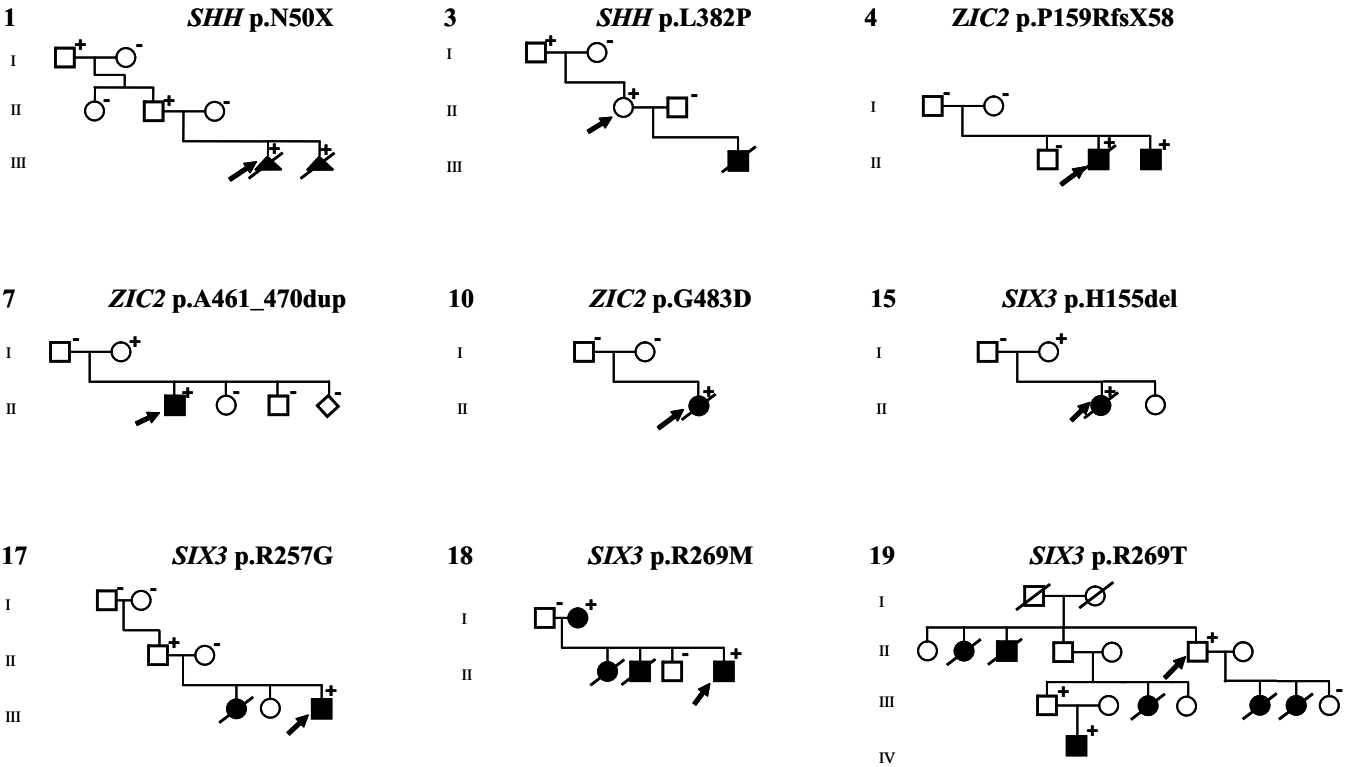
SIX2

Figure 2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SHH p.L382P
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7
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10
ZIC2 p.G483D
15
SIX3 p.H155del
17
SIX3 p.R257G
18
SIX3 p.R269M
19
SIX3 p.R269T
I
II
III
IV

Figure 3