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

3
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20
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22 Competing Interest: None to declare

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

40

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

42

43

44 **INTRODUCTION**

45 Holoprosencephaly (HPE; MIM236100) is a structural anomaly of the human brain that occurs in early
46 embryonic life. The disorder originates around the fourth week of pregnancy when the prosencephalon
47 (forebrain) fails to cleave or incompletely cleaves into diencephalon and telencephalon, into distinct left
48 and right hemispheres and into olfactory and optic bulb tracts. The estimated prevalence of HPE is 1 in
49 16000 after birth, but the prevalence of HPE in early embryogenesis is much higher (1 in 250)¹.
50 Classification is based on severity of the brain malformation and ranges from the most severe alobar form
51 (single primitive ventricle, >60% of the cases), to the semi-lobar (incomplete separation of the ventricles,
52 around 25% of the cases) and the lobar form (complete separation with continuity across the cortex)^{2,3}.
53 Another mild form of HPE, called middle inter-hemispheric variant is characterized by failure of
54 separation of the posterior frontal and parietal lobes⁴. In many but not all cases the cerebral defects or
55 classifications correlate very well with the facial appearance, better known as “the face predicts the
56 brain”⁵. These facial appearances range from severe anomalies such as cyclopia, anophthalmia and
57 proboscis to mild such as midline cleft lip/palate, hypotelorism, flat nose, microcephaly and single
58 maxillary central incisor. Additionally, craniofacial malformations, neurological features, endocrine
59 disorders, oromotor dysfunction and dysautonomic dysfunction can be observed¹.
60 The etiology of HPE is very heterogeneous and both environmental as well as genetic causes are known
61 to be involved. Environmental factors, including maternal insulin-dependent diabetes mellitus, alcoholism
62 and exposure to drugs or infections are the primary cause for only a very small percentage of patients¹.
63 Approximately 25% of patients comprise monogenic syndromes in which HPE is one of the
64 malformations and about 40% of HPE patients harbor a chromosomal aberration⁶. Non-syndromic HPE in
65 the remaining 35% of patients is caused by single-gene defects with reduced penetrance. Several genes
66 have been identified to cause HPE including *SHH*⁷, *SIX3*⁸, *ZIC2*⁹, *TGIF*¹⁰, *GLI2*¹¹, *PTCH1*¹² and
67 *DISP1*¹³. In this study, we screened the four major genes known to be involved in HPE namely *SHH*,
68 *ZIC2*, *SIX3* and *TGIF* by sequence and MLPA analysis in a Dutch cohort of 86 sporadic and familial non-

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

71

72 **MATERIALS AND METHODS**

73 **Patient Population**

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

83

84 **PCR and sequence analysis**

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

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

95 coding sequences (Genbank cDNA accession numbers: *SHH*; NM_000193.2, *SIX3*; NM_005413.2, *ZIC2*;
96 NM_007129.2, *TGIF*; NM_003244.2) and mutations were confirmed on DNA extracted from the second
97 blood sample if available. The frequency of novel mutations was determined in a control population of
98 >100 healthy controls. Non-paternity was excluded in case of *de novo* mutations by multiplex VNTR
99 analysis using the AmpF ℓ ST $\text{\textcircled{R}}$ Identifiler PCR Amplification kit (Applied Biosystems, Foster City, CA).

100

101 **Multiplex Ligation-dependant Probe Amplification (MLPA) analysis**

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

108

109 **Affymetrix 250K *Nsp I* SNP array**

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

118

119

120 **RESULTS**

121 In total, we detected 21 mutations in 86 patients (24%) (table 1, supplementary table 2). The mutations
122 included 2 gene deletions, 2 nonsense mutations, 5 frame shift mutations, 1 in-frame deletion, 1 repeat-
123 expansion mutation (3x), 1 splice-site mutation and 7 missense mutations. Pathogenicity of mutations was
124 based on several features including the *de novo* character of mutations, segregation with clinical features
125 of HPE, exclusion of being a polymorphism (absence in control/HPE population), conservation and
126 location of mutation. A overview of all detected mutations with genetic and clinical features is depicted in
127 tables 1 and 2. A summary of all currently known mutations in the four genes, including this study, is
128 given in supplemental table 2. Some of our detected mutations were included in recent global studies to
129 study number, type and location of mutations. No other information such as sex, clinical characteristics,
130 inheritance, familial history etc was included in these studies¹⁴⁻¹⁶.

131 The distribution of the 21 mutations over the genes was 3 in *SHH* (14%), 9 in *ZIC2* (43%), 9 in *SIX3*
132 (43%) and 0 in *TGIF* (0%). Eleven (52%) mutations occurred *de novo* and non-paternity was excluded in
133 all of them.

134 ***SHH* mutations**

135 We detected 3 mutations in the *SHH* gene. Mutation c.148_166del19 (p.Asn50X) was detected in an
136 aborted male fetus (21⁺⁴ weeks) with (semi)lobar HPE, median cleft lip/palate, hypotelorism, an
137 underdeveloped nose and left isomerism of the lungs. The father showed microcephaly, hypoplastic alae
138 nasi, thin upper lip and long uvula and carried the mutation as well as the paternal grandfather. Also a
139 second pregnancy was terminated (14⁺³ weeks) due to HPE signs detected with ultrasound (cleft
140 lip/palate, hypotelorism, underdeveloped nose) and detection of the mutation in amniotic cells (pedigree
141 1/figure 2, picture A and B/figure 3). This couple underwent pre-implantation genetic diagnosis (PGD)
142 and delivered a healthy son in August 2008. Mutation c.304 T>C (p.Cys102Arg) was detected in an
143 aborted female fetus (23 weeks) with alobar HPE and arose *de novo*. The autopsy report listed facial
144 dysmorphism including hypotelorism and a flat nose. Mutation c.1145 T>C (p.Leu382Pro) was detected
145 in a 46-year-old female showing no HPE clinical characteristics. She was tested because her first child

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

149 **ZIC2 mutations**

150 We detected 9 mutations in the *ZIC2* gene. Mutation c.479delC (p.Pro159ArgfsX58) was detected in an 8-
151 year-old boy with lobar HPE, deafness, mental retardation and spastic diplegia. Both parents were healthy
152 and non-carriers but one older brother died of alobar HPE three weeks after birth. Cultured fibroblasts of
153 this deceased older brother showed that he was carrier of the same mutation (pedigree 4/figure 2)
154 suggesting germ line mosaicism. The mutation was not detected in semen of the father. Marker analysis
155 indicated that the risk haplotype, shared by the two affected brothers and not by a healthy brother, was
156 inherited from the mother (data not shown). Mutation c.1090 C>T (p.Gln364X) occurred *de novo* and was
157 detected in a female first child with semilobar HPE including microcephaly, hypotelorism and a very slow
158 general development (picture C1-C3/figure 3). Mutation c.1325delC (p.S442SfsX112) occurred *de novo*
159 and was detected in an aborted female fetus with lobar HPE and no further facial anomalies. The
160 previously described alanine tract expansion mutation c.1377_1406dup30 (p.A461_470dup) was detected
161 in three unrelated families: In the first family (7430) the mutation was detected in a male child with
162 semilobar HPE, microcephaly, mental retardation, diabetes insipidus and tetraparesis. The boy does not
163 speak and does not walk at currently age 8. The asymptomatic mother with a normal brain MRI carried
164 the expansion (pedigree 7/figure 2, picture D, E, F/figure 3). In the second family (12940) the alanine
165 tract occurred *de novo* and was detected in an aborted male fetus (23⁺⁴ weeks) with semilobar HPE
166 (picture G/figure 3) and no apparent facial dysmorphism except for a broad mouth. In the third family
167 (14475) the expansion also occurred *de novo* and was detected in an aborted female fetus (30⁺⁶ weeks)
168 with semilobar HPE without further external malformations (picture H/figure 3). Mutation c.1448 G>A
169 (p.G483D) was found in an aborted female fetus (14 weeks) with alobar HPE in combination with an
170 encephalocele (pedigree 10/figure 2). The mother also carried the mutation but further clinical
171 examination of the mother showed no microsigns of HPE. She refused an MRI or further familial genetic

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

179 ***SIX3* mutations**

180 We detected 9 mutations in the *SIX3* gene. Mutation c.235 A>G (p.M79V) occurred *de novo* and was
181 detected in an aborted female fetus (18⁺³ weeks) with alobar HPE and microcephaly, a single nostril, flat
182 face and a malformed upper lip without cleft. *De novo* mutation c.404_407dupGCGC (p.V137RfsX18)
183 was detected in a 10-month-old girl with semilobar HPE (partial hypoplasia of the corpus
184 callosum)(picture K/figure 3). Mutation c.463_465delCAC (p.H155del) was found in a baby girl who
185 died shortly after birth due to HPE, bilateral cleft lip and palate and a single nostril. The mutation was
186 also detected in the asymptomatic mother (pedigree 15/figure 2). Mutation c.736delA (p.T245fsX5)
187 occurred *de novo* in a baby girl that died 12 days after birth because of alobar HPE with a single nostril,
188 flat nose, median cleft lip/palate and hypotelorism. Mutation c.769 C>G (p.R257G) was detected in a 16-
189 year-old boy with lobar HPE, microcephaly and mental retardation. A younger sister died the second day
190 after birth. The autopsy report on the sister did not indicate specific HPE characteristics as the forebrain
191 was completely divided and the corpus callosum was present. There were however brain abnormalities
192 including brain atrophy, absent nervi olphactorii and large ventricles. DNA of the sister was not available.
193 The asymptomatic father carried the mutation, but his parents did not (pedigree 17/figure 2). Mutation
194 c.806 G>T (p.R269M) was detected in a 21-year-old man (picture L/figure 3, photo taken at teenage
195 years) with semilobar HPE cleft lip/palate, diabetes insipidus, epileptic fits and severe mental retardation
196 (pedigree 18/figure 2). Clinical diagnosis of HPE was made at age 4¹⁷. Two younger children of this
197 family died shortly after birth due to HPE but DNA of them was not available¹⁷. The mother has a solitary

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

213

214 DISCUSSION

215 Screening the four major HPE genes (*SHH*, *SIX3*, *ZIC2* and *TGIF*) in 86 Dutch index patients and 54
216 family members led to the detection of 21 heterozygous mutations (24.4%). This mutation rate is
217 comparable to previous reports for non-syndromic HPE¹⁸. Only the alanine tract expansion in the *ZIC2*
218 gene and the c.404_407dupGCGC mutation in the *SIX3* gene have been identified previously^{9,19,20}. Of the
219 31 family members tested for a familial mutation, 9 were carriers of whom 4 showed major or minor HPE
220 signs. Therefore around 55% of mutation carriers were completely asymptomatic.

221 We detected only 3/86 mutations in *SHH* (3.5%), which is significantly lower than the previously
222 reported 121/1100 (10.7%) by Roessler et al.¹⁵ (p=0.047, $\chi^2=3.94$). Two of the mutations were familial
223 and showed extremely wide phenotypic variability within the family as previously described¹⁸. The

224 p.N50X mutation truncates the SHH protein in the SHH-N domain, thereby eliminating the Hint motif
225 necessary for autocatalytic processing and is considered a biologically null allele¹⁵. Missense mutation
226 p.C102R is also located in the important SHH-N region of the protein and is very highly conserved in
227 other species up to *c. elegans*. Combined with the *de novo* character of this mutation, pathogenicity of this
228 mutation is a likely explanation for the alobar HPE. Missense mutation p.L382P is located in the SRR
229 region, in which several HPE mutations have been detected and which functions to bind cholesterol¹⁵.

230 We detected 9/86 mutations in *ZIC2* (10.5%), a percentage comparable to a recent summarizing study
231 from Roessler et al.¹⁶. Consistent with previous studies^{9,16,21} we found: 1) a high frequency (>55%) of
232 frame shifts, nonsense mutations and gene deletions (Table 1), 2) >50% *de novo* mutations, 3) several
233 frame shift mutations in the C-terminal end of the *ZIC2* protein, leaving the zinc-finger domains
234 theoretically intact, 4) the poly-alanine tract expansion as a prevalent mutation type. Inconsistencies with
235 previous studies were also found: in two of three patients the alanine tract expansion was *de novo*, in the
236 third patient maternally inherited, therefore not predominantly from the father as previously described.
237 Additionally, the mother was not a mosaic carrier of the mutation¹⁹. The one missense mutation we
238 detected, p.G483D, is located in the C-terminal end of the *ZIC2* protein where so far only frame shifts and
239 ins/del mutations have been detected. This mutation does not occur in a conserved region and the glycine
240 amino-acid is not well conserved among species. However, as the mutation was not observed in a panel of
241 110 healthy controls and was never described in literature (>1000 HPE index patients) it is very unlikely
242 to be a common polymorphism. The aborted fetus showed alobar HPE without facial anomalies and the
243 asymptomatic mother also carried the mutation. Since minor/absent facial dysmorphism combined with
244 severe brain malformations and incomplete clinical penetrance are specifically consistent with *ZIC2*
245 mutations, we consider this variant most likely pathogenic.

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

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

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

276 genes is evident as we detected two gene deletions, representing 12% of the total number of mutations
277 detected. Earlier, Bendavid et al.²⁴ already described several microdeletions in other genes (*SHH* and
278 *TGIF*) using the subtelomeric MLPA kit as well as precisely sized duplications and deletions at HPE loci
279 using array-CGH²⁵. It is therefore clear that complete gene deletions of all major HPE genes are possible
280 and should be included in diagnostic testing.

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

290

291 **CONFLICT OF INTEREST**

292 The authors declare no conflict of interest.

293

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296 cases.

297

298 **SUPPLEMENTAL DATA**

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

300

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426

427

428 **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 | <i>SHH</i> | 10304 | c.148_166del19 | p.N50X | Paternal | fetus (M) | A/B | | |
| 2 | | 10959 | c.304 T>C | p.C102R | <i>De novo</i> | fetus (F) | | | |
| 3 | | 14317 | c.1145 T>C | p.L382P | Paternal | 46 (F) | | | |
| 4 | <i>ZIC2</i> | 8774 | c.479delC | p.P159RfsX58 | Germline | 8 (M) | C | | |
| 5 | | 9004 | c.1090 C>T | p.Q364X | <i>De novo</i> | 0 (F) | | | |
| 6 | | 14914 | c.1325delC | p.S442SfsX112 | <i>De novo</i> | fetus (F) | | | |
| 7 | | 7430 | c.1377_1406dup30 | p.A461_470dup | Maternal | 1 (M) | | D/E/F | |
| 8 | | 12940 | c.1377_1406dup30 | p.A461_470dup | <i>De novo</i> | fetus (M) | | | G |
| 9 | | 14475 | c.1377_1406dup30 | p.A461_470dup | <i>De novo</i> | fetus (F) | | H | |
| 10 | | 14919 | c.1448 G>A | p.G483D | Maternal | fetus (F) | | I | |
| 11 | | 11643 | c.1508_1520del13 | p.G503AfsX48 | <i>De novo</i> | 1 (M) | | | |
| 12 | | 9723 | Gene deletion | - | unknown | 62 (F) | | J | |
| 13 | | <i>SIX3</i> | 12248 | c.235 A>G | p.M79V | <i>De novo</i> | | fetus (F) | K |
| 14 | | | 9823 | c.404_407dupGCGC | p.V137RfsX18 | <i>De novo</i> | | 1 (F) | |
| 15 | | | 10482 | c.463_465delCAC | p.H155del | Maternal | | 0 [†] (F) | |
| 16 | 8616 | | c.736delA | p.T245fsX5 | <i>De novo</i> | 0 [†] (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 | <i>De novo/germline</i> | 2 [†] (M) | | | |
| 21 | 12519 | | Gene deletion | - | <i>De novo</i> | fetus (M) | P | | |

429

430

431 **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 | <i>SHH</i> | 10304 | p.N50X | (semi)lobar | X | X | X | | X | | |
| 2 | | 10959 | p.C102R | alobar | X | | X | | X | | |
| 3 | | 14317 | p.L382P | None | | | | | | | |
| 4 | <i>ZIC2</i> | 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 | | <i>SIX3</i> | 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 | | |
| 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 | | | | | | | |

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

433 **TITLES AND LEGENDS TO FIGURES**

434 **Figure 1: Characterization of SIX3 gene deletion**

435 Family 12519 with the *SIX2/3* deletion originally detected with MLPA and confirmed with by
436 250K Affymetrix SNP array (194 SNP's =>1.45 Mb). The deleted area is highlighted and
437 zoomed in with the *SIX2/3* genes at the centromeric end of the deletion.

438

439 **Figure 2: Pedigrees of familial mutations**

440 Family pedigrees according to numbering of Tables 1 and 2 are indicated at the top left corner of
441 each pedigree. Specific mutation for the family is indicated in the right upper corner.

442 + indicates mutation carriers; - indicates non-mutation carriers; squares indicates males, circles

443 females; filled symbols are patients with HPE clinical characteristics; arrows indicate family

444 index.

445

446 **Figure 3: Pictures of patients**

447 Pictures of HPE patients or their relatives. Letters below the pictures are referred in the text as

448 well as in table 1. All photographs are published with informed consent of index patients or their

449 parents.

450

451

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

| Gene | Exon | Primer sequence 5'→3' | Amplicon length | cDNA length | PCR mix |
|-----------------|-----------------|---|--|-------------|-----------------|
| <i>SHH</i> | 1 | F: <u>TGTA AACGACGGCCAGTGGGAAGGAGCGGGT</u> GAAATC R: CAGGAAACAGCTATGACCGAGAGCGCACACGCACACAC | 514 | 451 | VS standard |
| | 2 | F: <u>TGTA AACGACGGCCAGTGGCACCTCACAGGGCAGGTT</u> R: CAGGAAACAGCTATGACCGGTGTGTCTGGGCCATTCCA | 577 | 262 | VS standard |
| | 3A | F: <u>TGTA AACGACGGCCAGTGTGCGGCGCCACAAAGAG</u> R: CAGGAAACAGCTATGACCCCAGCCAACCTCTGTGACCG | 526 | 269 | VS standard |
| | 3B [#] | F: <u>TGTA AACGACGGCCAGTACGGCGCCAAGAAGGTCTT</u> R: CAGGAAACAGCTATGACCCCAGCTGTGCTCCTCGAT | 381 | 381 | VS ¹ |
| | 3C [#] | F: <u>TGTA AACGACGGCCAGTGCCCGGGCCAGCGCGTGTACGTGG</u> | 313 | 313 | VS ² |
| | 3D | F: GCAGAGTAGCCCTAACCG R: CATAACAAACAAATAAATAGCCAG | 438 | 195 | Invitrogen |
| | <i>ZIC2</i> | 1A | F: CTGGAGCCCGGTGGCCGCC R: CGGCGTGGGGCCCGAGCG | 407 | 307 |
| 1B [#] | | F: <u>TGTA AACGACGGCCAGTCGCACATGGGAGCCTTCAA</u> R: CAGGAAACAGCTATGACCCGGCTGCTGCCATGTTCA | 522 | 522 | VS standard |
| 1C [#] | | F: <u>TGTA AACGACGGCCAGTGGCGCAACTCCACAACCAGT</u> R: CAGGAAACAGCTATGACCAGTCTCAGCCTGTGCGTGTCTC | 514 | 455 | VS standard |
| 2 [#] | | F: <u>TGTA AACGACGGCCAGTCAGAGGGGATCGGACTGGTT</u> R: CAGGAAACAGCTATGACCAGCACCCAGAAATGTTCC | 436 | 164 | VS standard |
| 3 | | F: GGGGAACATTTCTGGGGGTG R: ATCACAAGGTGCCCTCGCTGCT | 547 | 444 | Invitrogen |
| <i>SIX3</i> | | 1A | F: GGTGTGGGTGTCCCTTACGCC R: GCAGCTGGAACATGGACAACCTCTT | 424 | 424 |
| | 1B | F: ACCACCGCTCCATACTTCTG R: TGTGGTTCTCAAGGATGTGG | 399 | 399 | Invitrogen |
| | 1C [#] | F: <u>TGTA AACGACGGCCAGTCTTCCACACGGGCAACTTCC</u> R: CAGGAAACAGCTATGACCCTCCGACTGGCCAGGAGTCA | 514 | 390 | VS standard |
| | 2 | F: CGTACTTTGTCGCTTGCACC R: CGGTTTGTCTGGGGATGG | 497 | 311 | Invitrogen |
| <i>TGIF</i> | 2 | F: <u>TGTA AACGACGGCCAGTACTCTGACAGCGCCGAGGTG</u> R: CAGGAAACAGCTATGACCGGCGCTGAGCGTGTCTTCTG | 588 | 184 | VS standard |
| | 3 | F: <u>TGTA AACGACGGCCAGTGGCATTGGTTGAGAGCCTCC</u> R: CAGGAAACAGCTATGACCTGACTTGACACACAGGAATGCC | 510 | 227 | VS standard |
| | 4A | F: <u>TGTA AACGACGGCCAGTGCTTGCTTCTTGCGGAGC</u> R: CAGGAAACAGCTATGACCTGGGTACATGAGAGAGGTGTCT | 581 | 405 | VS standard |
| | 4B | F: TCTCAGAACCCGTTGGCT R: TCATCTCTTGCTTACACC | 700 | 639 | VS standard |

453 [#] in house design, underlined: M13-sequence

454

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

456

457 ***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 |

458

459

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 |

460

461 **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 |

462

463 **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 |

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- 465 *SHH* Genbank accession NM_000193.2
 466 *SIX3* Genbank accession NM_005413.3
 467 *ZIC2* Genbank accession NM_007129.2
 468 *TGIF1* Genbank accession NM_003244.2
 469

Figure 1

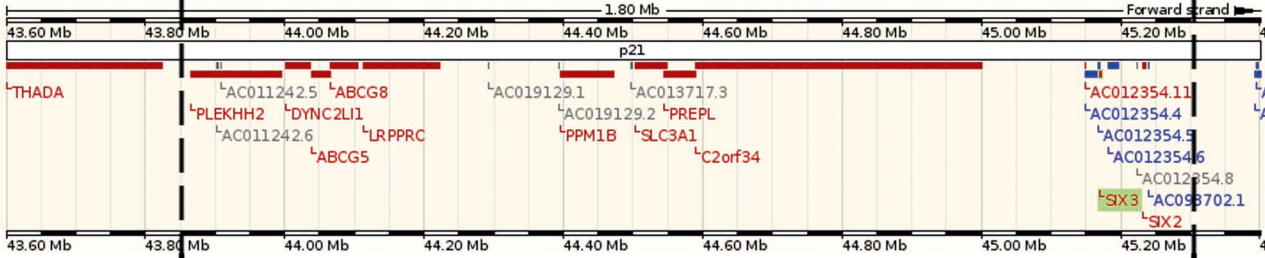
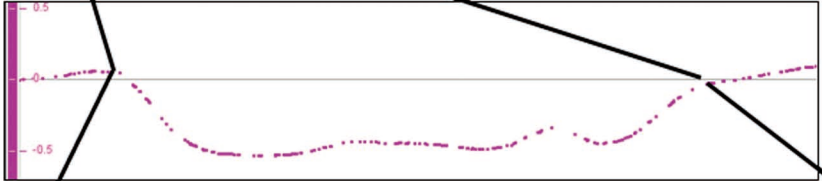
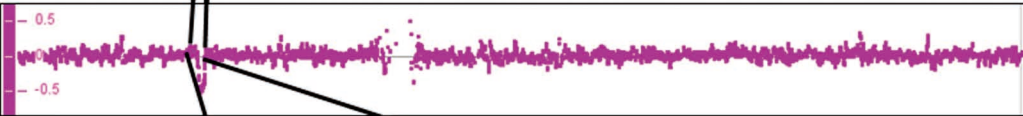


Figure 2

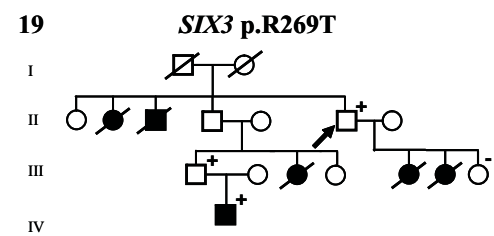
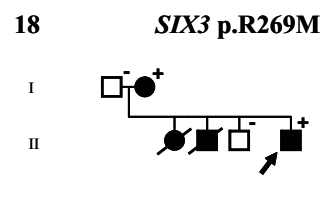
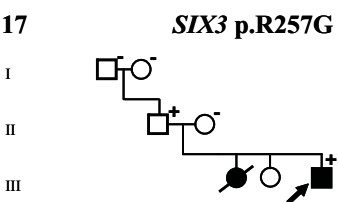
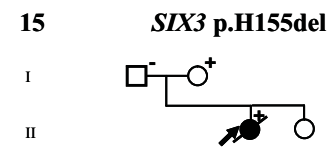
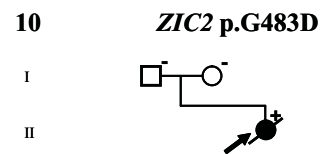
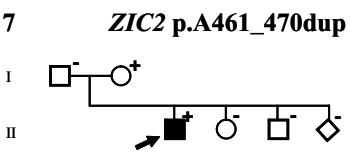
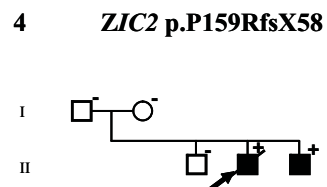
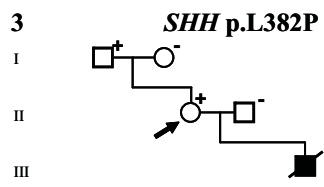
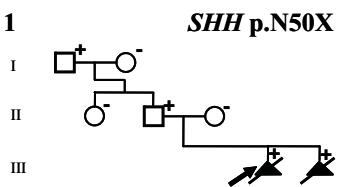


Figure 3

