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

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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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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)<sup>1</sup>.  
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)<sup>2,3</sup>.  
53 Another mild form of HPE, called middle inter-hemispheric variant is characterized by failure of  
54 separation of the posterior frontal and parietal lobes<sup>4</sup>. 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”<sup>5</sup>. 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 observed<sup>1</sup>.  
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 patients<sup>1</sup>.  
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 aberration<sup>6</sup>. 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*<sup>7</sup>, *SIX3*<sup>8</sup>, *ZIC2*<sup>9</sup>, *TGIF*<sup>10</sup>, *GLI2*<sup>11</sup>, *PTCH1*<sup>12</sup> and  
67 *DISP1*<sup>13</sup>. 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 $\ell$ 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 studies <sup>14-16</sup>.

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<sup>+4</sup> 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<sup>+3</sup> 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<sup>+4</sup> 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<sup>+6</sup> 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<sup>+3</sup> 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 4<sup>17</sup>. Two younger children of this  
197 family died shortly after birth due to HPE but DNA of them was not available<sup>17</sup>. 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<sup>+4</sup> 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 HPE<sup>18</sup>. Only the alanine tract expansion in the *ZIC2*  
218 gene and the c.404\_407dupGCGC mutation in the *SIX3* gene have been identified previously<sup>9,19,20</sup>. 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.<sup>15</sup> (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 described<sup>18</sup>. 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 allele<sup>15</sup>. 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 cholesterol<sup>15</sup>.

230 We detected 9/86 mutations in *ZIC2* (10.5%), a percentage comparable to a recent summarizing study  
231 from Roessler et al.<sup>16</sup>. Consistent with previous studies<sup>9,16,21</sup> 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 mutation<sup>19</sup>. 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<sup>1,19,22</sup>. 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.<sup>22</sup> 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.<sup>14</sup> 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)<sup>14,23</sup> and a high  
258 penetrance of clinical symptoms within familial mutation carriers. In a recent study Domené et al.<sup>20</sup>  
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)<sup>20</sup>. 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.<sup>24</sup> 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-CGH<sup>25</sup>. 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<sup>24,25</sup>  
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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295 We thank the patients and their families for participating in this study and all clinicians who referred  
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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**

<b>Nr</b>	<b>Gene</b>	<b>Fam nr.</b>	<b>Mutation</b>	<b>Amino-acid change</b>	<b>Mode of inheritance</b>	<b>Age at testing (Sex)</b>	<b>Picture (fig 3)</b>		
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 <sup>†</sup> (F)	
16	8616		c.736delA	p.T245fsX5	<i>De novo</i>	0 <sup>†</sup> (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 <sup>†</sup> (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)	(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: <u>CAGGAAACAGCTATGACCGGTGTGTCTGGGCCATTCCA</u>	577	262	VS standard
	3A	F: <u>TGTA AACGACGGCCAGTGTGCGGCGCCACAAAGAG</u> R: <u>CAGGAAACAGCTATGACCCAGCCAACCTCTGTGACCG</u>	526	269	VS standard
	3B <sup>#</sup>	F: <u>TGTA AACGACGGCCAGTACGGCGCCAAGAAGGTCTT</u> R: <u>CAGGAAACAGCTATGACCCAGCTGTGCTCCTCGAT</u>	381	381	VS <sup>1</sup>
	3C <sup>#</sup>	F: <u>TGTA AACGACGGCCAGTGCCCGGGCCAGCGCGTGTACGTGG</u>	313	313	VS <sup>2</sup>
	3D	F: GCAGAGTAGCCCTAACCG R: CATAACAAACAAATAAATAGCCAG	438	195	Invitrogen
	<i>ZIC2</i>	1A	F: CTGGAGCCCGGTGGCCGCC R: CGGCGTGGGGCCCGAGCG	407	307
1B <sup>#</sup>		F: <u>TGTA AACGACGGCCAGTCGCACATGGGAGCCTTCAA</u> R: <u>CAGGAAACAGCTATGACCCGGCTGTGCCATGTTCA</u>	522	522	VS standard
1C <sup>#</sup>		F: <u>TGTA AACGACGGCCAGTGGCGCAACTCCACAACCAGT</u> R: <u>CAGGAAACAGCTATGACCAGTCTCAGCCTGTGCGTGTCTC</u>	514	455	VS standard
2 <sup>#</sup>		F: <u>TGTA AACGACGGCCAGTCAGAGGGGATCGGACTGGTT</u> R: <u>CAGGAAACAGCTATGACCAGCACCCAGAAATGTTCC</u>	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 <sup>#</sup>	F: <u>TGTA AACGACGGCCAGTCTTCCACACGGGCAACTTCC</u> R: <u>CAGGAAACAGCTATGACCCTCCGACTGGCCAGGAGTCA</u>	514	390	VS standard
	2	F: CGTACTTTGTCTGCTTGCACC R: CGGTTTGTCTGGGGATGG	497	311	Invitrogen
<i>TGIF</i>	2	F: <u>TGTA AACGACGGCCAGTACTCTGACAGCGCCGAGGTG</u> R: <u>CAGGAAACAGCTATGACCGGCGCTGAGCGTGTCTTCTG</u>	588	184	VS standard
	3	F: <u>TGTA AACGACGGCCAGTGGCATTGGTTGAGAGCCTCC</u> R: <u>CAGGAAACAGCTATGACCTGACTTGACACACAGGAATGCC</u>	510	227	VS standard
	4A	F: <u>TGTA AACGACGGCCAGTGCTTGCTTCTTGCGGAGC</u> R: <u>CAGGAAACAGCTATGACCTGGGTACATGAGAGAGGTGTCT</u>	581	405	VS standard
	4B	F: TCTCAGAACCCGTTGGCT R: TCATCTCTTGCTTACACC	700	639	VS standard

453 <sup>#</sup> 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**

<b>Mutation</b>	<b>Amino-acid change</b>	<b>Literature</b>
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
<b>c.148_166del19</b>	<b>p.N50X</b>	<b>This study,<sup>15</sup></b>
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
<b>c.304 T&gt;C</b>	<b>p.C102R</b>	<b>This study,<sup>15</sup></b>
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

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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

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c.1130 T>C	p.F337S	15
c.1132_1140del9	p.A378_F380del	26
c.1142 G>C	p.R381P	21
<b>c.1145 T&gt;C</b>	<b>p.L382P</b>	<b>This study,</b> <sup>15</sup>
c.1147 G>A	p.A383T	<sup>39</sup>
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

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**SIX3 mutations**

<b>Mutation</b>	<b>Amino-acid change</b>	<b>Literature</b>
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
<b>c.235 A&gt;G</b>	<b>p.M79V</b>	<b>This study,</b> <sup>14</sup>
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
<b>c.404_407dupGCGC</b>	<b>frameshift</b>	<b>This study,</b> <sup>20</sup>
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
<b>c.463_465delCAC</b>	<b>p.His155del</b>	<b>This study,</b> <sup>14</sup>
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
<b>c.736delA</b>	<b>frameshift</b>	<b>This study,</b> <sup>14</sup>
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
<b>c.769 C&gt;G</b>	<b>p.R257G</b>	<b>This study,</b> <sup>14</sup>
c.770 G>C	p.R257P	8
c.773 G>T	p.R258L	20
c.785 G>A	p.R262H	20
<b>c.806 G&gt;T</b>	<b>p.R269M</b>	<b>This study,</b> <sup>14</sup>
<b>c.806 G&gt;C</b>	<b>p.R269T</b>	<b>This study,</b> <sup>14</sup>
<b>c.806+1 G&gt;T</b>	<b>Alternative splicing</b>	<b>This study,</b> <sup>14</sup>
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
<b>Del SIX3</b>	<b>Gene deletion</b>	<b>This study</b>

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461 **ZIC2 mutations**

<b>Mutation</b>	<b>Amino-acid change</b>	<b>Literature</b>
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
<b>c.479delC</b>	<b>p.P160RfsX58</b>	<b>This study,</b> <sup>16,22</sup>
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	
<b>c.1090 C&gt;T</b>	<b>p.Q364X</b>	<b>This study,</b>	<b>16,22</b>
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	
<b>c.1325delC</b>	<b>p.S442SfsX112</b>	<b>This study</b>	
c.1329delC	p.S444AfsX111	22	
c.1330_1365del	p.S444_455del	19,44	
c.1366_1395dup30	p.A456_465dup	22	
<b>c.1377_1406dup30</b>	<b>p.A461_470dup</b>	<b>9,15,19, this study</b>	
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
<b>c.1448 G&gt;A</b>	<b>p.G483D</b>	<b>This study</b>
c.1452_1456del5	p.A485RfsX43	16
c.1455_1461delinsCG	p.G487LfsX41	16
<b>c.1520del13</b>	<b>p.G503AfsX48</b>	<b>This study,<sup>16</sup></b>
c.1559delA	p.H520PfsX35	16
<b>Del ZIC2</b>	<b>Gene deletion</b>	<b>This study,<sup>22</sup></b>

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463 **TGIF mutations**

<b>Mutation</b>	<b>Amino-acid change</b>	<b>Literature</b>
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

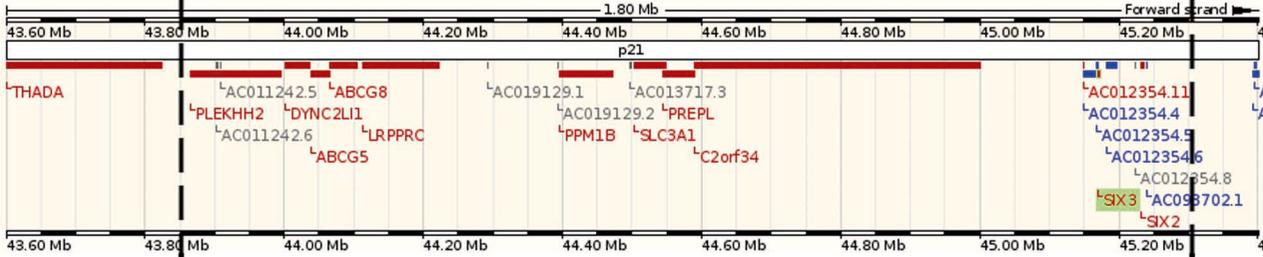
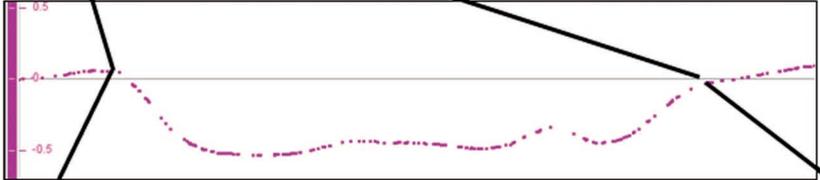
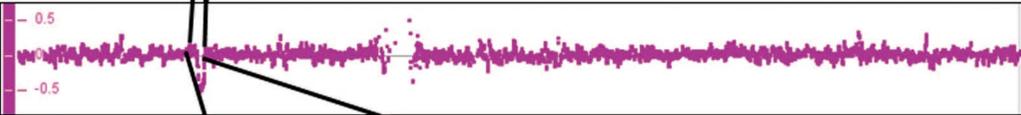


Figure 2

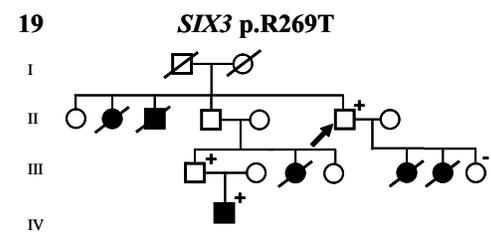
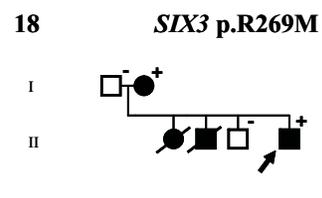
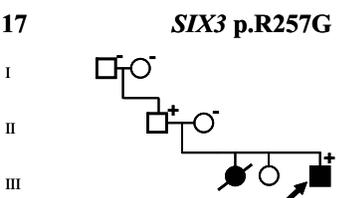
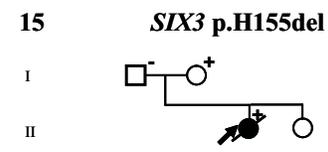
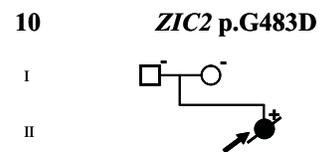
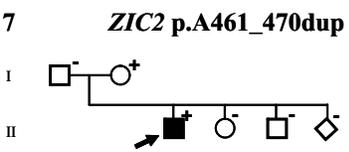
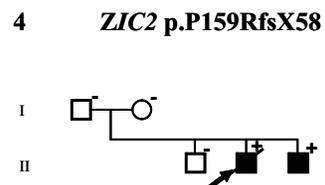
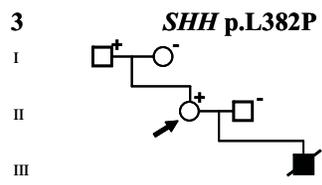
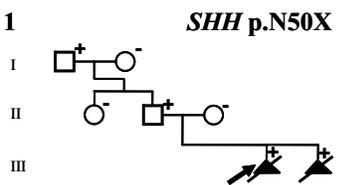


Figure 3

