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New findings for phenotype-genotype correlations in a large European series of holoprosencephaly cases

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ABSTRACT

Background:

Holoprosencephaly (HPE) is the most common forebrain defect in humans. It results from incomplete midline cleavage of the prosencephalon.

Methods:

We report a large European series of 645 HPE probands (and 699 relatives), consisting of 51% foetuses and 49% liveborn children.

Results:

Mutations in the four main genes involved in HPE (*SHH*, *ZIC2*, *SIX3*, *TGIF*) were identified in 25% of cases. The *SHH*, *SIX3* and *TGIF* mutations were inherited in more than 70% of these cases, whereas 70% of the mutations in *ZIC2* occurred *de novo*. Moreover, rearrangements were detected in 22% of the 260 patients screened by array-CGH. Fifteen probands had two mutations providing additional support for the "multiple-hit process" in HPE. There was a positive correlation between the severity of the brain malformation and facial features for *SHH*, *SIX3* and *TGIF*, but no such correlation was found for *ZIC2* mutations. The most severe HPE types were associated with *SIX3* and *ZIC2* mutations, whereas microforms were associated with *SHH* mutations. We focused on the associated brain malformations, including neuronal migration defects, which predominated in individuals with *ZIC2* mutations, and neural tube defects, which were frequently associated with *ZIC2* (rachischisis) and *TGIF* mutations. Extra-craniofacial features were observed in 27% of the individuals in this series (up to 40% of those with *ZIC2* mutations) and a significant correlation was found between renal/urinary defects and mutations of *SHH* and *ZIC2*.

Conclusions:

We propose an algorithm based on these new phenotype-genotype correlations, to facilitate molecular analysis and genetic counselling for HPE.

INTRODUCTION

Holoprosencephaly (HPE; MIM#236100) is the most common brain malformation in humans. It results from incomplete midline division of the prosencephalon between days 18 and 28 of gestation.[1][2] The estimated prevalence of HPE in liveborn children is less than 1/10,000, but the incidence may be as high as 1/250 in first-trimester conceptuses.[3, 4] HPE is usually associated with facial abnormalities, such as cyclopia, proboscis, or cleft lip/palate in cases of severe HPE. It has been said that “the face predicts the brain” but this rule is not applicable in all cases.[5, 6] Three classical anatomical classes have been described, in increasing order of severity: lobar, semi-lobar and alobar HPE. The full spectrum of HPE also includes middle interhemispheric variants (MIH) or syntelencephaly, septopreoptic HPE and microforms characterised by midline defects (e.g. single maxillary median incisor (SMMI) or hypotelorism) without the brain malformations typical of HPE.[7, 8, 9, 10]

Not only is HPE phenotypically highly variable, its aetiology has also been shown to be very heterogeneous.[11, 12, 13] Indeed, HPE may be caused by chromosome abnormalities, such as trisomy 13, 18 and triploidy in particular, or may be one of the components of a multiple malformation syndrome, such as Smith-Lemli-Opitz or CHARGE syndrome. HPE may also result from exposure to maternal diabetes or hypocholesterolaemia during gestation.[14, 15] Four major genes responsible for HPE have been characterised — *SHH*, *ZIC2*, *SIX3* and *TGIF* — and another nine seem to play a lesser role in the occurrence of HPE.[13] The “multiple-hit hypothesis” is now the most widely accepted model of HPE. According to this hypothesis, combinations of mutations in major and/or minor HPE genes lead to the occurrence of HPE and may account for variability in terms of severity.[16] Genetic counselling thus remains very complex for families affected by HPE.[17] There are clearly many interactions between the various signalling pathways involved in forebrain development that remain to be elucidated.

It is therefore difficult to establish phenotype-genotype correlations for this disease, due to the large number of interacting factors. Studies of large series are therefore required. We and others have reported specific findings, including, in particular, the description of particular microforms often associated with *SHH*, the predominance of severe forms in subjects with *SIX3* mutations and a *ZIC2*-specific phenotype with minor facial features.[6, 9, 18, 19, 20, 21]

We report here clinical and molecular data for the largest ever European series, including 645 probands and their relatives and a high proportion of foetuses; only one larger series has been described, based solely on children with HPE (NIH, Muenke's team).[21] We aimed to identify new phenotype-genotype correlations in an updated large series of probands with at least one mutation in an HPE-related gene and/or at least one rearrangement identified on array-CGH analysis. Our statistical analyses confirm previous reports and focus, for the first time, on additional features, such as associated brain malformations, neural tube defects and extra-craniofacial defects. These new phenotype-genotype correlations should help to guide molecular analysis strategies and, thus, genetic counselling.

PATIENTS AND METHODS

In 1996, a European network for HPE was set up in Rennes, France.[1, 9, 18, 22] Clinical data for about 1500 patients, including 645 HPE probands and 699 relatives, were analysed and DNA was prospectively collected. All the data were input into a specific secure online database.

This study was approved by the institutional review board. All samples and clinical data were collected after informed consent had been obtained.

Molecular analyses

The four major HPE-associated genes (*SHH*, *ZIC2*, *SIX3* and *TGIF*) were routinely analysed by D-HPLC and/or direct sequencing in probands. The mode of inheritance was determined in cases in which samples from the parents were available. We analysed the *GLI2* gene in 208 HPE patients selected on the basis of a pituitary gland defect.[23, 24, 25]

We also searched for microcytogenetic rearrangements by multiplex ligation probe amplification (MLPA) (MRC-Holland) on the four genes. Array-CGH was performed with the Agilent Human Genome Microarray 44A and 244A kits (Agilent Technologies, Santa Clara, CA), for 260 patients.[26]

Clinical data

We collected data relating to facial morphology and associated features. This study focused on HPE patients without known chromosomal abnormalities that could be picked up by standard cytogenetic analysis and without identified syndromes.

We defined four categories of facial abnormality, in descending order of medical severity: (1) very severe phenotypes, such as cyclopia, ethmocephaly (proboscis), cebocephaly; (2) premaxillary agenesis, cleft lip or palate and less severe eye abnormalities (coloboma, retinal dysplasia); (3) mild midface malformations, such as pyriform sinus stenosis and choanal stenosis; (4) the mildest abnormalities, such as hypotelorism, solitary median maxillary incisor (SMMI), other mild non-specific defects and normal faces.

Associated brain malformations not included in the typical spectrum of HPE were also considered. These malformations included, in particular, posterior fossa abnormalities, such as rhombencephalosynapsis (RES), cerebellar hypoplasia and agenesis, but also pituitary defects and gyral and neuronal abnormalities. Neural tube defects, such as myelomeningocele, rachischisis and spina bifida, were considered independently of the associated brain malformations.

Extracraniofacial defects were essentially classified into six groups: (i) *heart defects*; (ii) *visceral malformations* (abnormal lung lobulation, lung hypoplasia, thymus hypoplasia, adrenal hypoplasia, adrenal cytomegaly, common mesentery, single umbilical artery, diaphragmatic hernia, exomphalos, inguinal hernia, oesophageal atresia, intestinal stenosis); (iii) *genital malformations* (micropenis, hypospadias, external and internal genital organs); (iv) *renal/urinary abnormalities* (ureteral dilatation, renal hypoplasia); (v) *skeletal abnormalities* (sacral agenesis and costovertebral abnormalities); (vi) *abnormalities of the extremities* (club hand or foot, postaxial polydactyly, brachydactyly, clinodactyly or simian crease).

Statistical analysis

The percentages in this report were calculated for the patients with documented data, as exhaustive clinical data were not available for all cases.

Variables are expressed as numbers and percentages. Comparisons were based on Pearson's χ^2 test and Fisher's exact tests, as appropriate. Correlations between ordinal variables were analysed by calculating Spearman's rank correlation coefficient and Goodman and Kruskal's gamma. We used a type I error of 0.05, by convention, and all analyses were studied carried out with R software version 2.12.2 (2011-02-25).[27]

RESULTS

The main data, including HPE type, facial abnormality category, associated brain malformations, neural tube defects and extracraniofacial malformations, are shown in table 1.

Table 1: Clinical data for the entire series, probands with alterations to the four main HPE-associated genes (*SHH*, *ZIC2*, *SIX3*, *TGIF*) and with abnormal array-CGH data.

Percentages are expressed according to the data recorded (500 probands for HPE type and face defect category) and correspond to the ratio of each feature reported in the probands of

the group concerned, with the exception of detailed extracraniofacial defects, which are expressed as a proportion of the total of extracraniofacial defects in each group. # Array-CGH was performed on 260 probands. In addition to the HPE types mentioned in the table, septo-optic dysplasia and atelencephaly were reported in 1.4% and in 0.6%, respectively, of the cases in this series. Statistical analyses were carried out by comparing the *SHH*, *ZIC2*, *SIX3*, *TGIF* subgroups and probands without modifications to any of the four genes. * Significant differences were found for of the distribution of HPE type ($p=3.8 \times 10^{10}$), face defect category ($p=9 \times 10^{10}$) and renal/urinary defects ($p=0.03$) in Fisher's exact tests.

The series

The series contained 645 probands, including 51% fetuses, and 699 relatives. The sex ratio of 1.2 (F:M) was consistent with a slight female predominance.

| Probands | HPE type | | | | | | Face defect category | | | | Associated brain malformations | Neural tube defect | % of probands | Extracraniofacial defects | | | | | |
|---------------------|---------------|-------------------------------|-------|------|-----------|-------|----------------------|---------|--------|-------|---------------------------------|--------------------|---------------|---------------------------|---------|---------------|----------|-------------|-----|
| | alobar | semilobar | lobar | MIH | microform | Cat I | Cat II | Cat III | Cat IV | Heart | | | | Visceral | Genital | Renal/urinary | Skeletal | Extremities | |
| Total cohort | 645 | 25% + 1% atelencephaly | 28% | 19% | 2% | 25% | 27% | 19% | 14% | 40% | 13% | 4% | 27% | 19% | 23% | 11% | 11% | 8% | 27% |
| SHH | 67 (10.4%) | 28%* | 34%* | 4%* | 0%* | 34%* | 38%* | 22%* | 26%* | 14%* | 9% | 5% | 24% | 8% | 24% | 8% | 28%* | 16% | 16% |
| ZIC2 | 53 (8.2%) | 30%* | 45%* | 15%* | 8%* | 2%* | 8%* | 8%* | 3%* | 81%* | 21% (15% neuronal migration) | 9% | 40% | 14% | 20% | 17% | 14%* | 9% | 26% |
| SIX3 | 33 (5.1%) | 52%* + 7% atelencephaly | 15%* | 11%* | 0%* | 15%* | 45%* | 24%* | 10%* | 21%* | 7% | 7% | 24% | 0% | 22% | 11% | 0%* | 22% | 45% |
| TGIF | 11 (1.7%) | 43%* | 14%* | 29%* | 0%* | 14%* | 55%* | 27%* | 0%* | 18%* | 9% | 9% | 27% | 25% | 50% | 12% | 0%* | 0% | 13% |
| CGH | 56* (22%) | 19% | 37% | 17% | 0% | 27% | 8% | 38% | 8% | 46% | 11% | 4% | 26% | 19% | 22% | 17% | 11% | 11% | 20% |

Systematic molecular analyses showed point mutations or deletions in the four major genes involved in HPE (*SHH*, *ZIC2*, *SIX3* and *TGIF*) in 25.4% of the subjects (164 probands; Figs. 1 and 2)[6, 18, 21, 28, 29, 30] More fetuses than children were found to carry an HPE-associated gene deletion (68% vs 32%). Previous array-CGH analysis showed rearrangement rates as high as 25% in an initial study of 120 patients.[26] We describe here an additional 140 undergoing screening, with rearrangements identified in 22% (n=56) of the total of 260 probands. (Table 1)

Most severe cases of HPE were assigned to facial defect category 1 or 2, whereas most of microforms were classified as category 3 or 4 (Fig. 3). As expected, category 4 included all cases of MIH and, surprisingly, a relatively high proportion of the cases of alobar or semilobar HPE. RES, the equivalent of HPE in the cerebellum, was described in six HPE cases and schizencephaly was reported as potentially associated in only three cases.[31] Extracraniofacial features were observed in 70 cases, including 13 cases of multiple congenital malformations mostly concerning abnormalities of the extremities, such as club hand or foot, simian crease, postaxial polydactyly (5 cases) and visceral malformations, frequently with lung defects. Furthermore, three cases of situs inversus were observed.

An HPE spectrum disorder was reported in 79 of the 699 relatives (29% in parents and 71% in other relatives). All affected parents were diagnosed with brain microforms, which accounted for 44% of the abnormalities in these 79 relatives. More severe forms were reported in other relatives, such as siblings or cousins: 26% alobar HPE, 13% semi-lobar HPE and 7% lobar HPE. Facial abnormalities were correlated with HPE severity: 26% category 1, 13% category 2, 10% category 3 and 51% category 4. Associated brain malformations were observed in 6% of the affected relatives and neural tube defects were found in 8%. Extracraniofacial malformations were reported in 16% of relatives, including only two cases of multiple congenital malformations.

SHH

SHH mutations were found in 67 probands (10.4%) and in 47 relatives from 59 different families, confirming that *SHH* was the gene most frequently mutated in HPE. An HPE spectrum disorder was described in 13 relatives: 1 lobar case and 11 microforms (1 non documented). We found that 23% of the parents with *SHH* mutations presented a microform. Brain MRI revealed the presence of midline defects, such as an abnormal corpus callosum in the mother of one proband.

The sex ratio (F:M) was more balanced in *SHH* probands than in the series as a whole (0.9). The four categories of facial features were evenly distributed and microforms were, above all, associated with pyriform sinus and choanal stenosis (74% category 3). The proportion of coloboma cases was relatively high (15% of the series as a whole). Associated brain malformations mostly concerned posterior fossa abnormalities (7%). Extracraniofacial abnormalities included five cases of multiple congenital malformations, mostly visceral and renal/urinary defects. Sacral agenesis was also observed (associated in one case with costovertebral abnormalities) in several probands with large 7q36 deletions, including the *HLXB9* gene in particular (Currarino syndrome, MIM#176450).

ZIC2

ZIC2 mutations were identified in 53 probands (8.2%) and 14 relatives. Microforms were reported in 36% of the parents with *ZIC2* mutations (4/11). The sex ratio (F:M) of 1.8 was consistent with a marked predominance of female subjects. HPE tended to be alobar or semilobar (72%). Furthermore, only two HPE-related gene mutations were found in the 11 MIH cases in the total series, and both were *ZIC2* mutations.

The probands of the *ZIC2* group tended to have a combination of severe HPE with few facial features. Other specific findings were obtained for associated brain malformations, neural tube defects and extracraniofacial malformations. The neuronal migration abnormalities (15%) detected were lissencephaly, polymicrogyria or pachygyria in four cases and heterotopia in white matter and/or in the cerebellum in another four cases. One foetus with lobar HPE had heterotopia and gyration abnormalities among other multiple congenital anomalies (rachischisis, cleft palate, visceral and extremity abnormalities). Neural tube defects were reported in five cases, including two cases of rachischisis: one combined with pseudoanencephaly and the other with anencephaly with facial clefting in a monozygous twin

previously reported by Lazaro *et al.*[9] Both the cases of rachischisis in the overall series were found to have a *ZIC2* mutation. A high proportion of extracraniofacial abnormalities, mostly visceral abnormalities and abnormalities of the extremities, was associated with *ZIC2* mutations, including eight cases of multiple congenital malformations.

SIX3

SIX3 mutations were identified in 33 probands (5.1%) and 21 relatives. Microforms were found in three of the 17 parents. The sex ratio was in favour of a higher proportion of females. Very severe HPE, such as alobar HPE and atelencephaly, was described in 57% of cases. In one family, a missense mutation was found in a mother and her three foetuses with alobar HPE or atelencephaly.[32, 33]

Probands with *SIX3* mutations mostly had severe facial features (category 1 or 2). Severe ophthalmological defects were often reported with *SIX3* mutations, as shown by a high percentage of category 1 abnormalities, and coloboma was found in two category 2 cases. Associated brain malformations were mentioned in only 6% of the probands with *SIX3* mutations, including one case of gyrus abnormality associated with heterotopia. Finally, only one case of multiple congenital malformations was reported, with no heart defects among the extracraniofacial features.

TGIF

TGIF mutation was found in only 11 probands (1.7%) and eight relatives, including five parents with no reported HPE spectrum disorder. The sex ratio (1.3) was similar to that for the series as a whole. HPE tended to be of the alobar type, and facial defects were mostly severe: 82% of probands were classified as category 1 or 2. Associated brain malformation

was reported in only one case of ectopic posterior pituitary gland. The extracraniofacial abnormalities were not specific (one case of multiple congenital malformations).

GLI2

Evidence for a minor role of *GLI2* was provided by the very low rate of mutations in this gene in HPE spectrum disorders. Indeed, *GLI2* mutation was found in only three probands (1.4% in selected patients) and three relatives. Two of these missense mutations may be involved in the disorder but the pathogenicity of the third mutation could not be established with certainty. Samples from the parents were available for one patient, and analysis of these samples showed the mutation to be inherited. The brain malformations observed were lobar, semi-lobar and alobar HPE with facial abnormalities of category 2 or 4 (one undocumented case).

Interestingly, one foetus presented the combination of a *GLI2* missense mutation, inherited from an asymptomatic mother, with a *de novo* 13q deletion including the *ZIC2* gene. The proband had alobar HPE with complex heart malformations, bilobulated lung, gallbladder agenesis, hypospadias, no thumb or first metacarpal and unguis hypoplasia.

Microrearrangements

Array-CGH analysis identified rearrangements in 22% (56 cases) of the 260 probands. These rearrangements had arisen *de novo* in 14% and were inherited in 8%. They consisted of 45 isolated microdeletions (80%), including one ring chromosome and 19 microduplications (20%), with double rearrangements in eight cases. The rearrangement included one of the main HPE genes (*SHH*, *ZIC2* or *TGIF*) in eight probands. Recurrent rearrangements, involving *MACROD2* (20p) in seven cases and *PATCHD3* (10p) in six cases, were found to occur at a higher rate than in the general population.

The 56 probands with rearrangements included 59% fetuses and 41% liveborn children, with a balanced sex ratio (1.04). HPE types and facial features were evenly distributed, but with a higher frequency of semilobar forms or microforms and of category 2 (38%) or 4 (46%) than in the global series (Table 1; Fig 3). Extracraniofacial malformations included four multiple congenital abnormalities with no marked association.

Finally, we report seven cases in which a deletion or duplication was associated with a single mutation in an HPE-related gene (*SHH*, *ZIC2* and *SIX3*) (Table 2). The three fetuses and four children concerned had either isolated or syndromic HPE, with very various phenotypes in terms of HPE type and facial features.

Table 2: Clinical and molecular data for the 8 probands with double mutations: rearrangement detected on array-CGH and HPE gene mutation. The inheritance of rearrangements and mutations is reported in all cases in which molecular analysis of the parents of the proband was possible. The GenBank accession numbers are NM_00193.2 for *SHH* cDNA, NM_007129.2 for *ZIC2* cDNA, NM_005413.2 for *SIX3* cDNA, NM_003244.2 for *TGIF* cDNA and NM_005270.4 for *GLI2* cDNA.

| Proband no. | Foetus (F) / Child (C) | Sex | Form | HPE type | Facial defects category | Rearrangement | | Mutation (inheritance) |
|-------------|------------------------|-----|-----------|-----------|-------------------------|--------------------------------|------------------|--|
| | | | | | | Type (inheritance) | Significant gene | |
| 1 | F | M | syndromic | semilobar | 2 | Dup 3p14.3 (paternal) | | <i>SHH</i> c.388G>T (paternal) |
| 2 | F | M | isolated | lobar | 2 | Del 10p12.1 (paternal) | <i>PATCHD3</i> | <i>SHH</i> c.305G>A (paternal) |
| 3 | C | M | isolated | microform | 2 | Del 10p12.1 | <i>PATCHD3</i> | <i>SHH</i> c.1040C>G (<i>de novo</i>) |
| 4 | C | F | isolated | microform | 3 | Del 2q13 | <i>NPHP1</i> | <i>SHH</i> c.707G>A (maternal) |
| 5 | C | F | isolated | semilobar | 4 | Del 21q21.1 (<i>de novo</i>) | | <i>ZIC2</i> c.1025_1026delAA (<i>de novo</i>) |

| | | | | | | | | |
|---|---|---|-----------|-----------|---|---------------------------------------|----------------|---|
| 6 | C | F | isolated | semilobar | 3 | Del 20p12.1 | <i>MACROD2</i> | <i>SIX3</i> c.760T>G (maternal) |
| 7 | F | M | syndromic | alobar | 4 | Del 13q31.1 q34 (<i>de novo</i>) | <i>ZIC2</i> | <i>GLI2</i> c.2717C>G (maternal) |

Statistical analyses

We carried out statistical analyses to identify significant phenotype-genotype correlations. The population of probands with rearrangements was highly heterogeneous. We therefore decided to focus on probands with mutations in *SHH*, *ZIC2*, *SIX3*, *TGIF*, comparing these probands with those with no mutation in these genes.

Proportions of fetuses and children

As expected, the fetuses were significantly more likely to have severe types of HPE, whereas the liveborn children tended to have microforms ($p < 0.0001$). However, although there were more fetuses with HPE-associated gene deletions or array-CGH rearrangements, these differences were not statistically significant ($p = 0.09$ and $p = 0.2$, respectively).

HPE types

Studies of the distribution of HPE types between the five subgroups provided evidence for a highly significant correlation ($p < 0.0001$). Alobar HPE tended to be associated with *SIX3*, semilobar HPE with *ZIC2* and microform with *SHH* mutations. Lobar HPE did not seem to be particularly associated with any specific HPE gene in these analyses.

Facial defect categories

Facial features were studied as a function of the severity of the brain malformation. The findings for the *ZIC2* subgroup differed significantly from those for the other genes, as no

correlation between the HPE severity type and the severity of facial defects category was found (Fig. 3).

We also analysed the facial defects categories independently. A significant difference in the distributions of categories 1 and 4 was found, with a low frequency of probands with *ZIC2* mutations in category 1 and a very high frequency of such probands in category 4. A significant correlation was also found between *SHH* microforms and category 3 ($p=0.01$).

Brain-associated malformations; neural tube defects

No significant overall correlation was found between associated brain malformations or neural tube defects and molecular data, although these defects did appear to be more frequent in individuals with *ZIC2* and *TGIF* mutations ($p=0.18$).

Extracraniofacial defects

A correlation between renal/urinary malformations and certain mutations ($p=0.03$) was found. Renal/urinary defects were significantly more frequent in probands with *SHH* and *ZIC2* than in the probands of other subgroups. No significant difference was found in the distribution of the other extracraniofacial defects.

DISCUSSION

HPE is a very complex disorder in terms of both clinical variability and genetic signalling pathways. We report here the clinical and molecular data for the largest ever European series, encompassing 645 probands and 699 relatives, with a higher proportion of fetuses (51%) than in previous series.[19, 20, 21] The large number of patients included in this series resulted in sufficient power for the detection of statistically significant phenotype-genotype correlations.

In the series as a whole, the sex ratio (F:M) was 1.2, in favour of a slight predominance of female subjects. Female subjects were in the minority among probands with *SHH* mutations, for which the sex ratio was 0.9, whereas a high predominance of female subjects (sex ratio of 1.8) was observed among those with *ZIC2* and *SIX3* mutations. The *SIX3* sex ratio was consistent with that reported by Lacbawan.[19] This suggests that mutations in *ZIC2* or *SIX3* may be embryonic-lethal in males.

Screening for mutations in the major genes *SHH*, *ZIC2*, *SIX3* and *TGIF* and the minor gene *GLI2* in this large series gave an estimated global mutation rate of 25.8%, with specific mutation rates of 10.4% (*SHH*), 8.2% (*ZIC2*), 5.1% (*SIX3*), 1.7% (*TGIF*) and 0.4% (*GLI2*). These results are consistent with our previous findings for smaller series.[1, 9, 18, 34] Point mutations in *SHH*, *SIX3* and *TGIF* were highly heritable, with heritabilities of 73%, 88% and 100%, respectively, whereas most *ZIC2* mutations were *de novo* (70% of cases).[6, 20] This may be due to a higher penetrance and the high frequency of severe HPE in individuals with *ZIC2* mutations, resulting in the identification of a larger number of sporadic cases. By contrast to what was reported by Lacbawan *et al.*, no predominance of maternal inheritance was observed for *SIX3* mutations in our series, whereas such a pattern of inheritance was observed for *SHH* mutations.[19]

Overall, more severe types of HPE (atelencephaly, alobar or semilobar HPE) were reported in individuals with *SIX3* (74%) and *ZIC2* (75%) mutations than in individuals with *SHH* (62%) and *TGIF* (57%) mutations. Moreover, atelencephaly/aprosencephaly was associated exclusively with *SIX3* mutations. Pasquier *et al.* reported this particular association and other teams have reported a high proportion of severe cases in subjects with *SIX3* mutations, including ophthalmological defects in particular.[19, 20, 33] Thus, alobar HPE tends to be associated with *SIX3*, semilobar HPE with *ZIC2* and microforms with *SHH*, consistent with the findings of Solomon.[21] Furthermore, our findings confirmed the specific association of

MIH or syntelencephaly with *ZIC2*, as the mutations identified in four of the 11 cases of MIH in the series concerned the *ZIC2* gene.[6]

This study highlights the considerable intra- and interfamilial phenotypic variability in *SHH* probands.[35] We report here, for the first time, the high prevalence of brain microforms (74%) in probands with *SHH* mutations presenting, essentially, pyriform sinus and choanal stenosis (category 3). Associations with midline defects, coloboma and sacral agenesis (systematically linked to a large 7q36 deletion) were also observed. By contrast, facial morphology in subjects with *ZIC2* mutations was generally characterised by moderate facial dysmorphism or even a normal face (category 4), associated with alobar or semilobar HPE. This feature seems to be specific to *ZIC2* mutations and has also been reported by other teams.[6, 20] Solomon *et al.* mentioned a particular facial phenotype found in some of the cases in our series, with a high proportion of abnormal noses (9 cases) usually short, sometimes flat or large, and ear dysplasia (4 cases). Furthermore, statistical analyses revealed a lack of correlation between the severity of HPE brain malformation and facial features in probands with *ZIC2* mutations, but not in those with *SHH*, *SIX3* and *TGIF* mutations.

We also considered other associated brain malformations, focusing in particular on RES, which was associated with HPE in six of the 645 HPE probands. This extremely rare malformation is the histological equivalent of HPE for the cerebellum. The signalling pathways involved in this disease has yet to be identified, but we suggest that there may be interactions with HPE signalling pathways, as a *ZIC2* mutation has been reported in two siblings with partial RES and HPE.[36, 37] *SHH* and *SIX3* mutations have recently been associated with schizencephaly, but only three cases of schizencephaly were reported in our series, with no mutation identified in the probands concerned.[31]

We also identified two other previously unknown associations with *ZIC2* mutations: rachischisis and neuronal migration abnormalities. Neural tube defects are frequently reported in HPE, but rachischisis seems to be more specifically associated with *ZIC2* mutation, as such mutations were found in the only two cases of rachischisis in our series. In addition, neuronal migration abnormalities have already been described, but without phenotype-genotype correlations.[2, 38, 39] We focus here on neuronal migration abnormalities, such as gyral pattern abnormalities and heterotopia, which were almost exclusively restricted to probands with *ZIC2* mutations (8 cases). Hahn *et al.* found subcortical heterotopic grey matter or cortical dysplasia in two thirds of MIH cases.[38] In our series, neuronal migration abnormalities were observed in two of 11 cases of MIH, but no *ZIC2* mutation was identified in these cases.

We report here, for the first time, the frequency and variety of extracraniofacial malformations in a large series, together with the corresponding molecular data. Molecular defects were more frequent in *ZIC2* than in the other three main genes. We found a higher frequency of extracraniofacial defects than reported by Solomon, probably because his study focused on children, rather than fetuses.[6] Renal/urinary defects were significantly associated with *SHH* or *ZIC2* mutations and no such defects were found in probands with *SIX3* or *TGIF* mutations. *SIX3* was the only gene for which no heart defect was reported.

The data for *TGIF* and, above all, for *GLI2* revealed no significant phenotype-genotype correlations, but alobar HPE with severe facial features (category 1) seemed to be more frequent in probands with *TGIF* mutations, although phenotypic variability was high. We suggest that phenotype-genotype correlations for *TGIF* and *GLI2* should be analysed in larger series. Another disease spectrum, characterised by anterior pituitary insufficiency and polydactyly, seems to be more specifically linked to *GLI2* mutations[23, 40] (Dubourg, unpublished data).

Several signalling pathways are known to be involved in HPE, and the various mechanisms underlying HPE may account for the observed phenotypic variability. Indeed, *SIX3* is involved in eye development, consistent with the eye defects observed in probands with *SIX3* mutations, and in ventral forebrain development, through regulation of the *SHH* signalling pathway. Furthermore, *ZIC2* significantly differs from the other HPE-related genes, as shown in phenotype-genotype correlations, and its product also acts by different mechanisms. Indeed, *ZIC2* is known to act both early stage, in upstream *SHH* signalling during mid-gastrulation, and at later stages, in the development of the dorsal telencephalon, potentially accounting for the specific occurrence of MIH and neural tube defects in *ZIC2* probands.

Array-CGH studies in this series confirmed the high frequency of rearrangements reported in a preliminary study.[26] Indeed, rearrangements were identified in 22% of the 260 patients studied (14% *de novo* and 8% inherited). The proportion of fetuses and the severity of HPE phenotype (extracraniofacial malformations included) were not significantly higher in probands with rearrangements than in the series as a whole, as might have been expected. The higher frequency of category 2 and 4 probands may reflect more common features, such as cleft lip or palate (category 2) or mild non-specific features (category 4). This population seemed to be heterogeneous and an understanding of the molecular basis of this heterogeneity should facilitate the definition of more homogeneous subgroups. Indeed, the high frequency of rearrangements was consistent with there being a large number of loci corresponding to new candidate genes. The recurrent regions included the *MACROD2* and *PATCHD3* loci, in particular. Furthermore, recurrent deletion 6qter led to the identification of *DLL1* as a new HPE-associated gene and of NOTCH as a new signalling pathway involved in HPE.[41]

It has already been suggested that HPE involves a multiple-hit process.[26, 42] Animal models have provided evidence of digenism, by implicating either the same or two different signalling pathways. Genetic background also played an important role in determining the

severity of the phenotype.[43, 44, 45] In humans, low penetrance or variable levels of expression in HPE multiplex families and reports of patients with two different mutations support this hypothesis.[16, 24, 46, 47] Our series included up to 15 cases of double mutation: eight cases of double rearrangements and seven cases of rearrangement associated with a mutation in an HPE-related gene (6 rearrangements associated with a mutation in *SHH*, *ZIC2* or *SIX3* and one *ZIC2* deletion associated with a *GLI2* mutation). This findings provide solid support for the multiple-hit hypothesis in HPE.

In conclusion, even if the identification of an HPE gene mutation cannot provide a precise prognosis, the new phenotype-genotype correlations identified here should facilitate the definition of a better molecular analysis strategy. We propose a new algorithm based on these correlations (Fig. 3). Evidence of double mutations in HPE probands provides support for the HPE multiple-hit hypothesis underlying the complexity of genetic counselling and research on this disorder. The known clinical variability of HPE also suggests an overlap between environmental and genetic mechanisms. A combination of clinical and functional studies should help to improve our understanding of the complex interactions between the various signalling pathways involved in HPE and brain development.

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Legends:

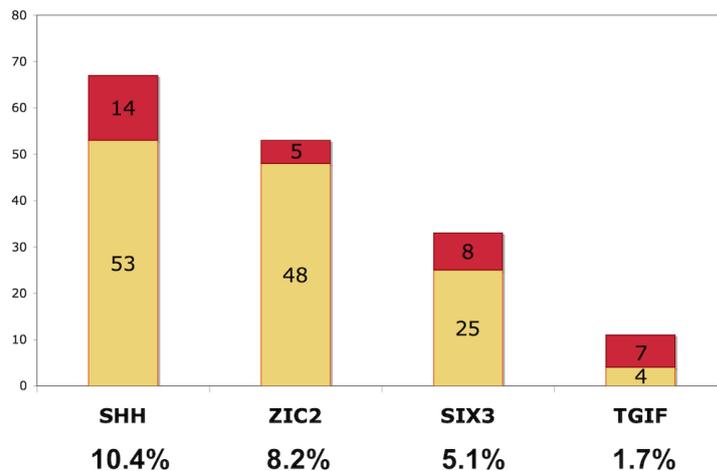


Figure 1: Schematic representation of point mutations and deletions in the *SHH*, *ZIC2*, *SIX3*, *TGIF* genes. Point mutations are represented in yellow and gene deletions are shown in red, with the corresponding number of probands. The percentage of probands (/645 probands) with a particular point mutation or a deletion is noted below each gene.

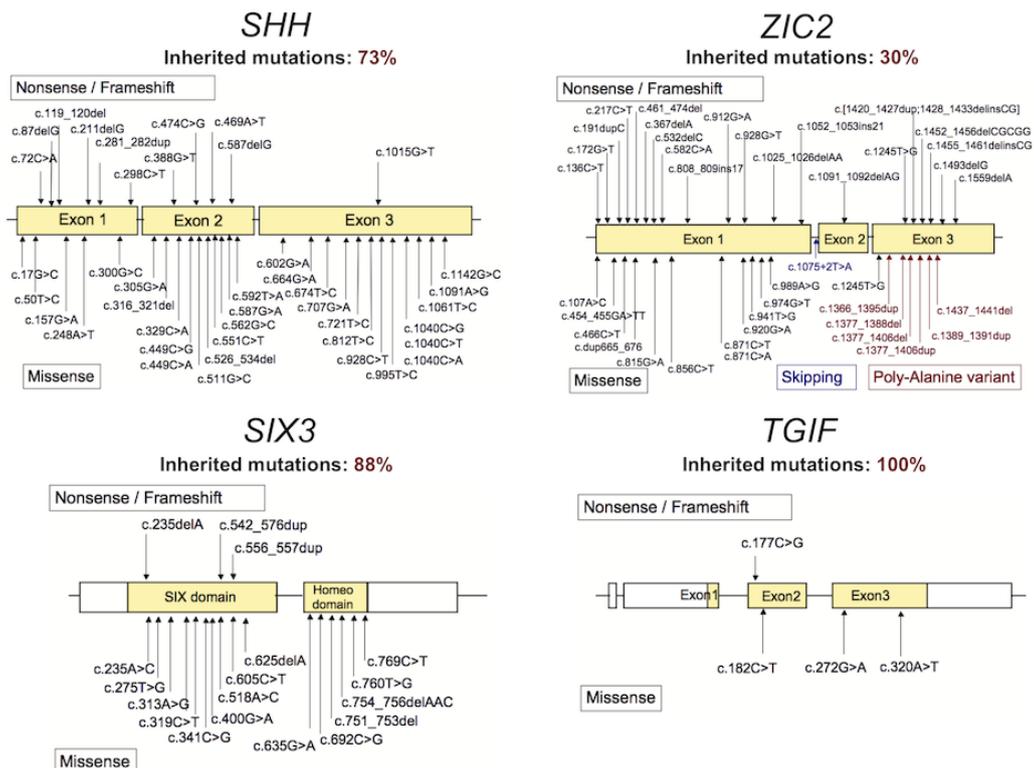
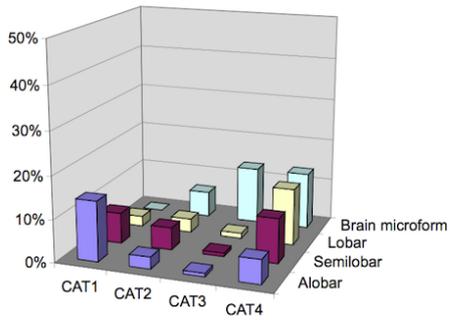
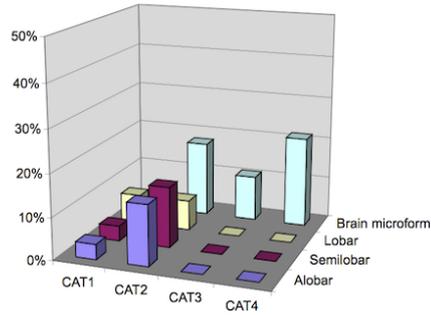


Figure 2: Point mutations and inheritance of the *SHH*, *ZIC2*, *SIX3* and *TGIF* genes. *SHH*: 41 different variations (73% missense, 27% nonsense or frameshift mutations); 73% inherited point mutations, predominantly of maternal (67%) rather than paternal (33%) origin. *ZIC2*: 44 different point mutations (29% missense, 55% nonsense or frameshift, 13% polyalanine tract, 3% splice alterations) with 70% occurring “*de novo*” and two cases of probable germline mosaicism. *SIX3*: 18 different point mutations (78% missense, 22% nonsense or frameshift). *TGIF*: 3 missense mutations and 1 nonsense mutation. The pathogenicity of these alterations has been confirmed by predictive or functional studies.[6, 18, 28, 29, 30] GenBank accession numbers are NM_00193.2 for *SHH* cDNA, NM_007129.2 for *ZIC2* cDNA, NM_005413.2 for *SIX3* cDNA and NM_003244.2 for *TGIF* cDNA.

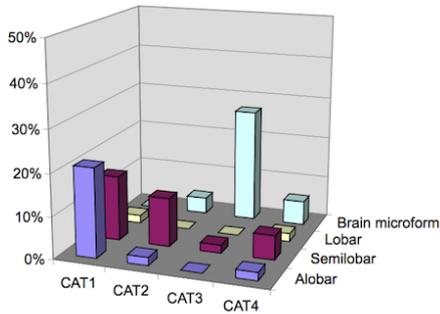
Entire series



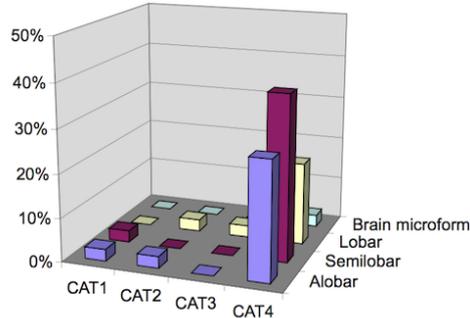
Microrearrangements



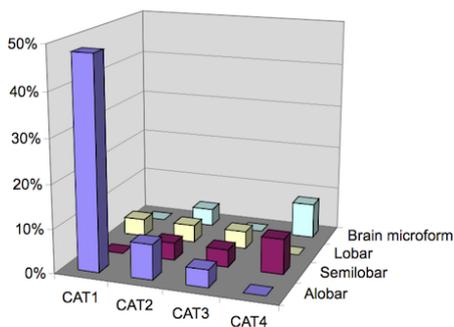
SHH



ZIC2



SIX3



TGIF

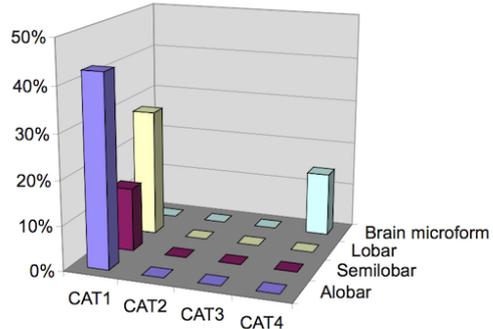


Figure 3: Face defect category, as a function of HPE type, for the series as a whole, probands with abnormal array-CGH findings and with altered *SHH*, *ZIC2*, *SIX3* and *TGIF* genes. The results presented are based on the data for 369 informative probands. Considering a positive gradient of severity from category 1 to category 4 in Spearman's rank correlation test, we found a significant correlation between the severity of HPE type and facial features for the entire series ($p < 0.001$) and for the *SHH* ($p < 0.001$), *SIX3* ($p < 0.001$) and *TGIF* ($p < 0.001$) subgroups, but not for the *ZIC2* subgroup ($p = 0.5$).

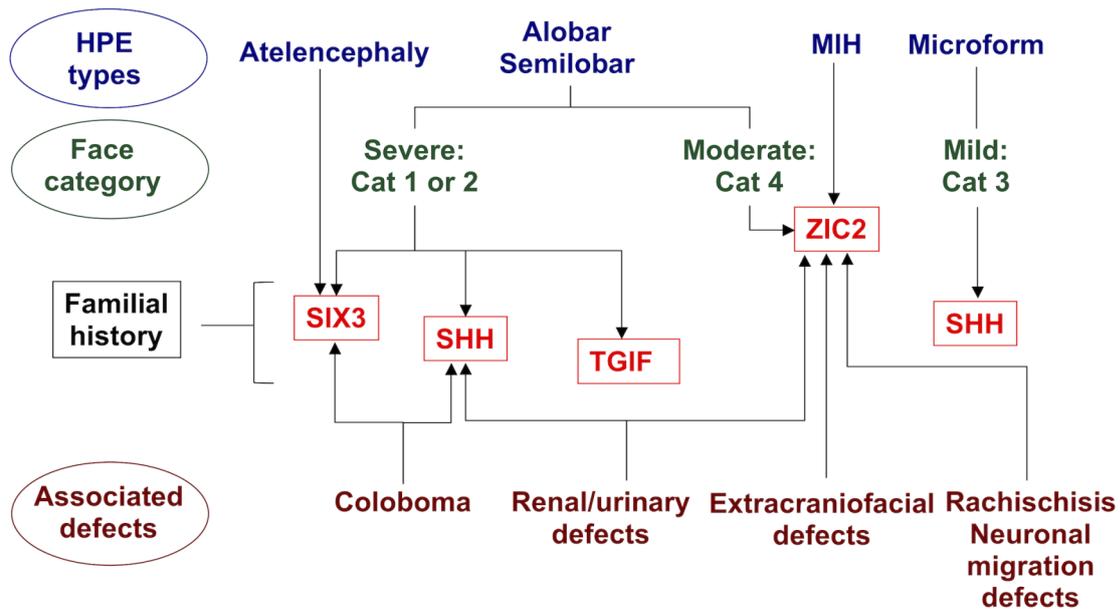


Figure 4: Algorithm for molecular analysis in HPE. In cases of familial HPE, analyses of *SHH*, *SIX3* or *TGIF* should be given priority over *ZIC2* analysis. Very severe forms of HPE, such as alobar and semilobar HPE, should lead to analyses of the *SIX3* (particularly in cases of atelencephaly/aprosencephaly) and *ZIC2* genes, but facial features may provide useful information. Indeed, probands with category 1 or 2 facial features associated with severe HPE, such as alobar or semilobar HPE, are more likely to display mutations in *SIX3* or *SHH*. By contrast, probands with severe brain malformations and category 4 facial features are likely to have mutations in *ZIC2*. No particular association with lobar HPE was found. Thus, if associated brain malformations are described, particularly in cases of neuronal migration abnormalities, and in cases of neuronal tube defects (rachischisis in particular), *ZIC2* gene analysis should be performed as a matter of priority. Moreover, *ZIC2* mutations are the principal cause of extracraniofacial malformations. Renal/urinary malformations are found preferentially in patients with *ZIC2* or *SHH* mutations and coloboma tends to be found in patients with *SHH* or *SIX3* mutations.

