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CONFLICT OF INTERESTS STATEMENT

All authors declare that they have no conflicts of interest in the research

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ABSTRACT

Holoprosencephaly (HPE) is the most common congenital cerebral malformation, characterized by impaired forebrain cleavage and midline facial anomalies. Heterozygous mutations in 14 genes have been associated with HPE, and are often inherited from an unaffected parent underlying complex genetic bases. It is now emerging that HPE may result from a combination of multiple genetic events, rather than from a single heterozygous mutation. To explore this hypothesis, we undertook whole exome sequencing (WES) and targeted high-throughput sequencing approaches to identify mutations in HPE subjects. We report here two HPE families in which two mutations are implicated in the disease. In the first family presenting two fetuses with alobar and semi-lobar HPE, we found mutations in two genes involved in HPE, *SHH* and *DISP1*, inherited respectively from the father and the mother. The second reported case is a family with a 9-year old girl presenting lobar HPE, harbouring two compound heterozygous mutations in *DISP1*. Together, these cases of digenic inheritance *SHH/DISP1* and autosomal recessive HPE suggest that in some families, several genetic events are necessary to cause HPE. This study highlights the complexity of HPE inheritance and has to be taken into account by clinicians to improve HPE genetic counseling.

KEY WORDS

DISP1, Holoprosencephaly, Multigenic Inheritance, *SHH*, Whole Exome Sequencing;

INTRODUCTION

Holoprosencephaly (HPE) is the most frequent cerebral malformation, with an occurrence of approximately 1 in 250 embryos and 1.31 in 10,000 births (1). HPE is characterized by a failure to define the midline of the forebrain and midface, with different degrees of severity from a lobar brain to alobar forms associated with cyclopia. Mild manifestations or microforms include ocular hypotelorism, microcephaly and a single central maxillary incisor (2). The mode of inheritance of HPE has been extensively discussed in the literature, and several genetic models have been proposed: autosomal dominant transmission, autosomal recessive transmission or association of mutations in multiple genes (2-5). All these studies point out a strong genetic heterogeneity, with several causative genes identified (Table 1). It mostly implicates heterozygous mutations in *SHH, ZIC2, SIX3* and *TGIF1,* which are considered as the four major genes involved in HPE. Heterozygous mutations in the minor genes *GLI2, PTCH1, DISP1, FOXH1, NODAL, TDGF1, CDON, GAS1, DLL1* and *FGF8* have been identified with a lower frequency (2, 6, 7). Recently, two recessive inheritance cases of HPE have been described, implicating mutations in the gene *STIL* (Table 1) (8, 9). Importantly, these genes are all involved in signalling pathways implicated in brain development (4, 9-16) and alteration of SHH signalling appears to be the most common cause of HPE (17).

Although the major genes have been formally involved, their penetrance is usually incomplete with an intra-familial phenotypic variability. Actually, mutations located in these genes are inherited from a parent, asymptomatic or displaying a microform of HPE, in 70% of the cases (2). For example, the same *SHH* mutation can be found in individuals harbouring either alobar HPE or minor forms (18). Consequently, the clinical variability could be due to abnormalities in other genes that have a function in the same or interacting signalling pathways (19, 20).

This is strongly supported by the description of mouse models carrying mutations in two genes of the same or different signalling pathways. For example, whereas *Gas1*-/- mutant mice exhibit partial fusion of the medial nasal processes and *Shh*+/- mice appear normal, *Gas1*-/-;*Shh*+/- mice embryos display complete fusion of the medial nasal processes (21), reminiscent of a HPE phenotype. Such examples of animal models are numerous and all

support the hypothesis that HPE could be due to a cumulative partial inhibition of signalling pathways implicated in forebrain development (6, 22).

However, there are only a few examples in the literature that suggest that HPE could be related to combined failures of several HPE genes in human (Table 2), including patients with co-occurring mutations or deletions in *SHH/TGIF1* and *SHH/ZIC2* (23). Nevertheless, sequencing of the four major genes (*SHH*, *ZIC2*, *TGIF1*, *SIX3*) in large HPE cohorts has not allowed to validate this hypothesis (2, 5, 14). Thus, the mode of inheritance of HPE is still unclear.

Identifying more genes in families in which polygenic inheritance is suspected would be very beneficial to understand the pathogenic mechanism of this developmental disorder. This is now facilitated by the recent development of next generation sequencing technologies (20). In this study, we performed whole exome sequencing (WES) in a family where the father carries a mutation in *SHH*, transmitted to 3 fetuses with semi-lobar and alobar HPE. We hypothesized that the fetuses have all inherited a second mutation in another gene from the mother. This original strategy was powerful as it revealed a second mutation in *DISP1* shared by the mother and the HPE fetuses. This family is the first one in which mutations in the two HPE-associated genes, *SHH* and *DISP1*, have been identified. Furthermore using a targeted NGS method, the involvement of *DISP1* in HPE was reinforced by the observation of two *DISP1* compound heterozygous mutations in another HPE family. These results support the complexity of HPE inheritance and raise important questions about how clinicians should consider the inheritance mode of HPE.

MATERIALS AND METHODS

Patients and samples

Patients presenting midline abnormalities and suspicion of holoprosencephaly were referred from the Centre Hospitalier Universitaire of Rennes (France) and the Hôpital Cochin (Paris, France) through the network of reference centres for developmental anomalies and malformation syndromes (CLAD centres). Patients and parents blood and tissue samples were obtained from the processing of biological samples through the Centre de Ressources Biologiques (CRB) Santé of Rennes BB-0033-00056 (http://www.crbsante-rennes.com). The research protocol was conducted under French legal guidelines and fulfilled the requirements

of the local institutional ethics committee. The parents of the photographed patient assented to include photographs in a scientific publication.

F1 family

The mother II2 had three terminations of pregnancy with fetuses (III1, III2 and III3) harbouring semi-lobar or alobar HPE (Fig. 1a and Table 3a). The F1 family also includes two other healthy girls III4 and III5, being 12 and 11 years old respectively. The father II1, the grandfather I1 and the uncle II3 all present microcephaly. This latter had 4 children among which 3 (III6, III7 and III9) harbour microcephaly and minor facial midline malformations (hypotelorism). The father II1 also had two healthy siblings, II5 and II6 (Table 3a). DNA was available for all family members except II4.

F2 family

The F2 family includes three members, the two healthy parents I1 and I2 and their 9-year old girl II1 who displayed a lobar HPE (Fig. 2 and Table 3b). She has two healthy siblings but their DNA was not available. Pregnancy was normal. A wide cleft palate was observed at birth, which was surgically treated. II1 was then referred to genetic counselling at 5-and-half-years old for psychomotor retardation (started walking at 21 months and had a language delay) and learning difficulties. On clinical examination, she weighed 16.5 kg (-1 standard deviation), was 109.5 cm tall, and had microcephaly (-2SD). She presented facial dysmorphy with flat face, short nose, small mouth and hypotelorism (Figs. 2c and 2d). At the sight of these clinical observations, we recommended a molecular diagnosis of HPE. Molecular testing of 18 HPE genes was performed on DNA of the daughter II1, revealing the presence of two compound heterozygous mutations in the minor HPE gene *DISP1*. Subsequently, to confirm the presence of HPE, MRI was performed and showed a mild form of lobar HPE with a very localized fusion of hemispheres in the forebrain (Fig. 2b).

Whole Exome Sequencing

WES was performed by the Genoscope on the DNA of the two parents II1 and II2 and one of the fetuses III2 in F1 family, using "SeqCap EZ Exome v3.0" capture (Roche) on HiSeq^{TM}2000 platform (Illumina). Exomes were homogenously sequenced with a mean coverage of 93% of the targeted bases with read depth greater than 20X, and an overall mean

depth of coverage of 112X. Bioinformatic analyses were conducted with the Illumina pipeline analysis CASAVA 1.8. The sequenced reads were aligned on the reference human genome 19 (hg19) with Eland v2.0 before variant calling (SNV and INDEL) with the CASAVA suite. Resulting variants were annotated using ANNOVAR v2.0 (http://www.openbioinformatics.org/annovar/annovar_filter.html). Variants population frequencies were extracted from three different public databases (the Exome Sequencing Project (ESP6500), the 1000 Genomes Project (1000g, 2014) and the Exome Aggregation Consortium (ExAC02)). Several bioinformatics predictions tools were used to predict conservation (GERP++, PhyloP, SiPhy, and PhastCons Elements 46-way) (24-27) and deleterious effect of SNVs and INDELs (SIFT, PolyPhen-2 HDIV and HVAR, LRT, Mutation Taster, Mutation Assessor, FATHMM, Radial SVM, LR and CADD) (28-34). Visual inspection of candidate variants was performed with Integrative Genome Viewer (IGV, Broad Institute).

Targeted high-throughput sequencing

Targeted NGS was performed using Ion Torrent technology (Life Technologies) on DNA from the girl II1 in F2 family. Two pools of 711 primer pairs were designed (Ion Ampliseq technology, Life Technologies) to sequence all the exons of a panel of 18 genes involved in HPE or candidates (*SHH*, *ZIC2*, *TGIF1, SIX3*, *DISP1*, *CDON*, *GAS1*, *SUFU*, *FGF8*, *FGFR1*, *NODAL*, *HHAT*, *SUFU*, *TDGF1*, *PTCH1*, *FOXH1*, *SOX2*, *DLL1*) and 2 *SHH* expression regulatory regions, spanning 111kb. Libraries were sequenced with Ion PGM™ System (Life Technologies). A PGM-specific pipeline incorporated in the Ion Torrent server (Torrent Suite version 4.0.2; Life Technologies) was used to perform the following steps: reads alignment on hg19, targeted regions coverage analysis, filtering and removal of poor signal reads. Variant calling was performed with the Ion Torrent Variant Caller version 4.0. Mutations were annotated using ANNOVAR v2.0 as described for WES analysis and with Alamut software (Interactive Biosoftware).

Sanger sequencing

Sanger sequencing in F1 and F2 families assessed the intrafamilial segregation of the candidate mutations found by NGS. This was done using the BigDye terminator cycle sequencing kit (Applied Biosystems) on an ABI3130xl sequencer (Applied Biosystems) and analysed using SeqScape software v2.6 (Life Technologies).

Functional validation of the *SHH* p.Pro347Gln mutation

The human *SHH* cDNA (RefSeq NM_000193) was cloned in a pMSCVneo (Clontech) vector. A mutated plasmid containing the *SHH* p.Pro347Gln mutation was obtained by site-directed mutagenesis using the QuikChange XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, USA). Plasmids containing cDNA of *SHH* WT or *SHH* p.Pro347Gln were transfected in C3H10T1/2 cells using Transfast (Promega). Six days later, the alkaline phosphatase activity was measured as previously described (35), reliable to the differentiation of C3H10T1/2 into osteoblasts under SHH action.

RESULTS

F1 family

In the F1 family, we showed by Sanger sequencing that a p.Pro347Gln mutation in *SHH* (c.1040C>A of RefSeq NM_000193) was present in fetuses III1, III2 and III3 inherited from the father II1 (Fig. 1a). It was inherited from the grandfather I1, and was also transmitted to the uncle II3, displaying microcephaly and hypotelorism. This uncle transmitted the *SHH* mutation to 3 children (III6, III7 and III9), also harbouring microcephaly and hypotelorism. WES analysis validated the known heterozygous *SHH* mutation in the father II1 presenting microcephaly and hypotelorism, and in the HPE fetus III2.

The deleterious effect of the *SHH* p.Pro347Gln mutation was evaluated using an adaptation of a cell-based assay previously described (35). The efficiency of SHH signalisation in presence of the mutation was evaluated by quantifying the SHH-dependent differentiation of mesenchymal cells (C3H10T1/2) into osteoblasts. This was assessed by measuring the activity of the alkaline phosphatase (ALP) in C3H10T1/2 cells, 6 days after transfection with the pMSCVneo plasmids containing either cDNA of *SHH* WT or cDNA of *SHH* p.Pro347Gln. The ALP activity of the cells expressing the mutated *SHH* was 0,04 µU by µg of protein extract, while it was 1.31 µU/µg in the protein extract from cells expressing *SHH* WT, meaning that C3H10T1/2 cells failed to undergo osteoblastic differentiation under action of mutated SHH. This strongly reflects the deleteriousness of the p.Pro347Gln mutation (Fig. 1b).

Based on the assumption that HPE observed in the fetus III2 resulted from the association of the *SHH* mutation with another mutation, variants were filtered according to this inheritance pattern. Only mutations inherited from the mother II2 were selected in the child III2. Intronic variants were filtered out, as well as synonymous variants and those that had a population allele frequency over 1% in any of the three public databases 1000g, ESP6500, and ExAC02. Thirty-four variants were selected, and a prioritization was performed using the cumulative predictions of ten bioinformatics tools. The 10 first-ranked candidate mutations are presented in supplementary data (Fig. S1). Most of these genes were already associated with genetic syndromes without any obvious link with forebrain development. We thus focused on a mutation in *DISP1*, known to be involved in HPE. This mutation is a substitution of a thymine in cytosine at location c.3287 of *DISP1* (NM_032890), leading to the change of a methionine in threonine at location p.1096. This mutation was listed as rs144673025 in dbSNP database, and has a minor allele frequency of 0,55% in ESP6500, 0,1398% in 1000g, and 0,6189% in ExAC02. Three bioinformatics prediction tools classified this mutation as deleterious (LRT, Mutation Taster, and FATHMM) while other tools predicted it as tolerated (PolyPhen-2, SIFT, radial SVM, LR, and Mutation Assessor) (Table 4).

We used Sanger sequencing to search for the *DISP1* c.3287T>C mutation in individuals III3, III4 and III5 (Fig. 1). DNA was no longer available to look for this *DISP1* mutation in fetus III1. A perfect co-segregation of the two mutations, *SHH* p.Pro347Gln and *DISP1* p.Met1096Thr was observed in HPE fetuses III2 and III3. Among the two healthy sisters, III4 has no mutation whereas III5 carries the *DISP1* mutation only.

F2 family

The F2 family was screened for 18 HPE candidate genes using targeted NGS for molecular diagnosis on the DNA of the daughter II1. Two heterozygous mutations were identified in the exon 10 of *DISP1*: the c.1087A>G transition leading to a missense mutation p.Asn363Asp and the c.1657G>A transition leading to a missense mutation p.Glu553Lys. Sanger sequencing on DNA of the parents showed that the p.Asn363Asp mutation was inherited from the father I1, and the p.Glu553Lys mutation was inherited from the mother I2 (Fig. 2a).

The *DISP1* p.Asn363Asp mutation was absent from public databases, and was predicted deleterious by 7 out of 10 of the prediction tools employed. The second mutation, p.Glu553Lys, was also predicted deleterious by the majority of the prediction tools, and was also absent from public sequencing databases (Table 4). According to the Alamut software

and the other conservation scores used, these two mutations were in highly conserved regions at both the nucleotide and the amino acid level.

DISCUSSION

The genetic heterogeneity of HPE is supported by reduced penetrance and the absence of obvious genotype-phenotype correlation. This is especially true for patients carrying heterozygous *SHH* mutations, as 45% harbor microforms, 45% present severe HPE and 10% are apparently asymptomatic (18). Using WES, we report here the first co-segregation of mutations in *SHH* and *DISP1* with severe HPE. Our results suggest that these mutations are combining to give a severe phenotype and provide strong evidence that digenic inheritance is a significant genetic model for HPE.

Relationships between SHH and DISP1 in HPE

In this study, we have investigated one family carrying a deleterious mutation in *SHH* and displaying variable expressivity of the disease. This mutation causes the change of proline in glutamine, which results in a severe reduction of SHH activity. Although microcephaly is not a typical sign of HPE, we can consider that the *SHH* mutation is responsible for this mild form of HPE in the present family (2). *SHH* mutation shows full penetrance with microcephaly whereas a second mutation in *DISP1* seems to be necessary to obtain a more severe HPE in 3 fetuses. This is also supported by the finding that the mother and a clinically normal sister carried only the mutation in *DISP1*.

In this study we also describe the first HPE case with compound heterozygous mutations in *DISP1*. Altogether, we find 3 different mutations (Fig. 3) in conserved regions of DISP1, including one in the Sterol Sensing Domain (SSD). The exact role of the SSD remains unclear, although most SSD containing proteins have been implicated in intracellular trafficking (36, 37). These 3 missense mutations in *DISP1* provide new arguments for the implication of this gene in HPE. Few other mutations in *DISP1* have been previously described in patient only harbouring microforms of HPE (38, 39). Noteworthy, all these *DISP1* mutations were inherited from clinically unaffected parents. This gives evidence that additional factors are necessary to potentiate these mutations of *DISP1* and to lead to HPE. As DISP1 mediates the secretion of SHH from producing cells, and allows consequent paracrine signalling (40), we hypothesized that these mutations have an impact on SHH signalling.

Mice data also strongly reveal the implication of *Disp1* in HPE (40-42). Heterozygous knockout for *Disp1* are undistinguishable from the wildtype whereas *Disp1*$^{-/-}$ embryos do not survive beyond E9.5 because of heart development defects. They also display cyclopia reminiscent to severe HPE (43). Further analysis of these mutants permitted to show that Shh signalling was disrupted in *Disp1*$^{-/-}$ embryos, indicating that *Disp1* is essential for proper Shh signalling. Thus, *Disp1* is most probably critical for ventral forebrain induction through its interaction with Shh pathway. Consequently, the co-segregation of a mutation in *SHH* and a mutation in *DISP1* with severe HPE strongly suggests that cumulative effects lead to severe impairment of forebrain development.

Inheritance in HPE

Some authors have proposed autosomal recessive inheritance in HPE (3, 44). However, despite systematic sequencing, homozygous mutations in the major HPE genes *SHH*, *ZIC2* and *SIX3,* were never described in HPE cohorts (2, 5, 45). Nonetheless some autosomal recessive cases implicating minor HPE genes (Table 1) have been reported. In 2007, a first case of recessive inheritance of *TGIF1* mutations was described, with the finding of two compound heterozygous mutations (46). A loss of function homozygous mutation in *FGF8* has also been identified in one consanguineous HPE family (47). More recently, hypomorphic alleles of *STIL* were implicated in two cases of autosomal recessive inheritance in HPE patients (8, 9). It was proposed that *STIL* had a function during early brain development linked to SHH signalling (9, 48). Here, we describe a first HPE patient displaying two mutated *DISP1* alleles whereas the two clinically normal parents carry only one mutated allele. This strongly supports that the presence of two mutations in a minor HPE gene exacerbates the risk of developing a HPE phenotype. Furthermore, the mild phenotype of the HPE patient described in this manuscript suggests that DISP1 activity is decreased such that it leads to a significant impairing of SHH pathway responsible for mild HPE, but above the threshold that would result in severe HPE and early lethality, as indicated by the mice model (43).

Currently, single heterozygous mutations are mainly reported in HPE cases (Table 1) and believed to account for the HPE phenotype (5). But still, it cannot be excluded that a mutation in another gene may underlie the observed forebrain defects. However, double heterozygous mutations for two HPE genes were rarely reported (Table 2). This is not really surprising because HPE genes are key developmental genes, and strong deleterious mutations in two of

these genes are probably not viable (44, 49). Thus, it is important to design the pipeline of WES analysis in order to avoid discarding mutations predicted to have a mildly deleterious effect in development genes. This strategy has enabled us to identify a new *DISP1* mutation in a family presenting two fetuses with alobar and semi-lobar HPE associated to a deleterious mutation in *SHH*. The identification of these two altered genes that have functional relationships in multiple affected individuals in one family strongly supports a digenic inheritance (19).

Polygenic inheritance has now been established for more and more other complex inheritance diseases among which digenic inheritance is the simplest form (19, 20). This mode of inheritance was reported in Kallmann syndrome (KS) (50), characterized by a defective hormonal reproductive axis and sense of smell. This developmental pathology was firstly described as autosomal dominant or X chromosome-linked. Further studies permitted to refine the genetics of KS syndrome by describing several patients harbouring two mutations in different genes (51, 52), combining major and minor KS genes, and giving evidence of a digenic inheritance of KS syndrome. This was also reported in patients with Alport syndrome, presenting mutations in two different collagen IV genes (*COL4A3* and *COL4A4*) (53). In some nephropathies, mutations in two genes encoding glomerular proteins nephrin and podocin (*NPHS1* and *NPHS2*) were identified in several patients (54). Such a multigenic inheritance is also well described in ciliopathies like Bardet-Biedl syndrome (BBS) with many patients harbouring mutations in two or more *BBS* genes (55). These cases illustrate how, in complex syndromes with variable severity, polygenic inheritance plays a role in the clinical expression of the disease. This is particularly relevant when there is functional relationships between mutated genes, as this is the case for HPE genes (44). A first online database dedicated to digenic diseases (DIDA, http://dida.ibsquare.be/) is now available (20). The cases described in our manuscript fully meet the required criteria to be included in DIDA database (20).

By describing new HPE families with no classical autosomal dominant inheritance, our work refines the genetic bases of HPE. This discovery has significant implications for genetic counseling especially for risk assessment of patient relatives. Clinical geneticists have to be aware of such different patterns of heritability, and WES or at least sequencing of a large panel of HPE genes, should be performed to establish a molecular diagnosis.

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LEGENDS

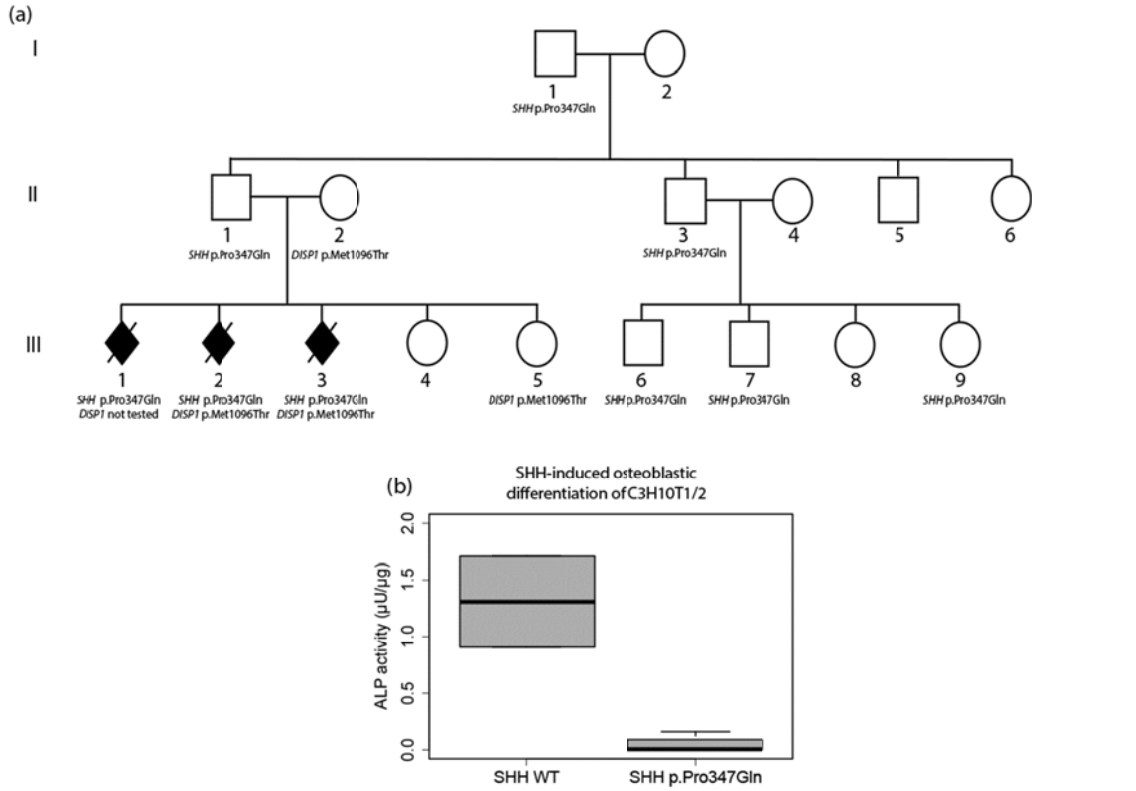

Figure 1. Pedigree of F1 family and functional test of the *SHH* p.Pro347Gln mutation

(a) Black symbols refer to HPE subjects. Mutations were represented in proteic nomenclature. The F1 family consisted of 17 members. Nine of them harbor the *SHH* p.Pro347Gln mutation, inherited from the paternal grand-father I1. In addition to the *SHH* mutation, fetuses III2 and III3 have a *DISP1* p.Met1096Thr mutation, transmitted by their mother II2. The fetus III1 was not tested for *DISP1*, as DNA quantity was insufficient. (b) Alkaline Phosphatase (ALP) activity measured in total protein extracts from C3H10T1/2 cells transfected with plasmids containing *SHH* WT or *SHH* p.Pro347Gln.

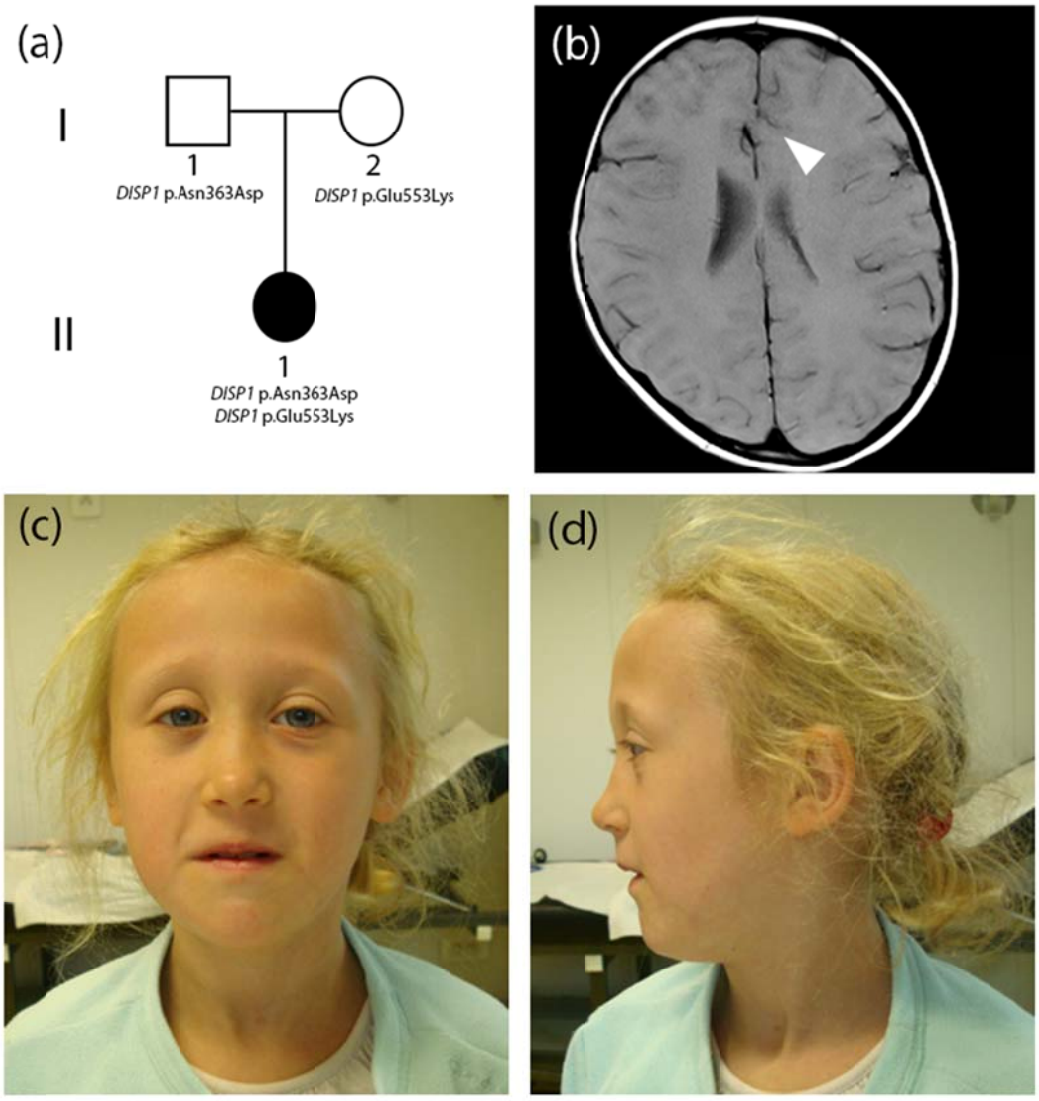

Figure 2. Pedigree of F2 family, and MRI of individual II1 of F2 family

(a) The F2 family consisted of 3 members. The daughter II1 has two *DISP1* compound heterozygous mutations, *DISP1* p.Asn363Asp, inherited from the father I1, and *DISP1* p.Glu553Lys, inherited from the mother I2. (b) Axial brain MRI of individual II1 of F2 family, harbouring a minor form of HPE, showing a very localized fusion of the hemispheres in the forebrain (white arrow). Facial (c) and lateral (d) photographs of the daughter II1 showing mild facial malformations.

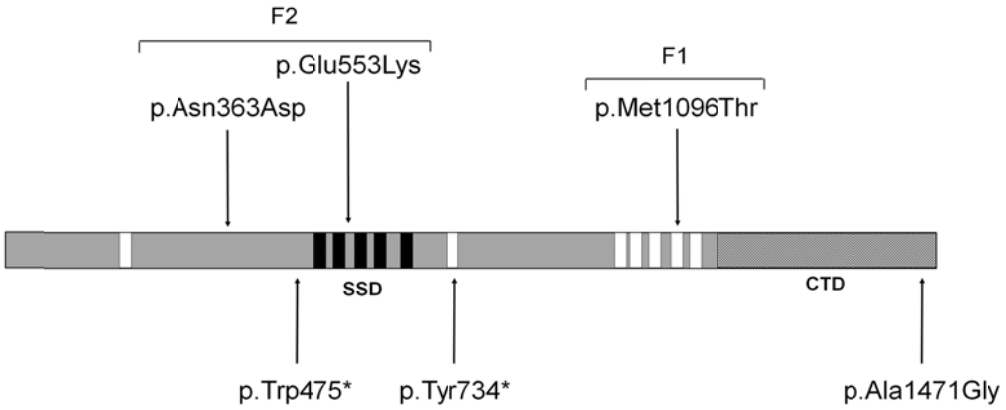

Figure 3. Distribution of all *DISP1* point mutations reported so far in HPE subjects

Mutations are shown on a schematic representation of DISP1 protein. The white and black rectangles represent transmembrane helical domains, including the Sterol-Sensing Domain (SSD) from amino acid 486 to 658 (black). The hatched area is the C-terminal Domain (CTD, 360 last aminoacids of the protein). Mutations presented by families F1 and F2 are represented at the top, whereas mutations previously reported in the literature are represented at the bottom. The p.Trp475* and p.Tyr734* mutations were described by Roessler et al (38). The first mutation was present in a girl with seizures, developmental delay, midline cleft lip/palate and mild decortication, inherited from her mother. The second mutation was transmitted from a mother to her daughter who presents facial malformations: bilateral cleft lip/palate, hypotelorism, upslanting palpebral fissures and solitary maxillary central incisor. The p.Ala1471Gly mutation was reported by in a boy, born with heart abnormalities (ventricular septal defect and abnormal aorta), Bochdalek congenital diaphragmatic hernia and left-sided cleft lip with bilateral cleft palate (39).

Table 1. Characteristics of genes and mutations implicated in holoprosencephaly.

The mutation frequencies are given in qualitative terms: “High” is for genes mutated in more than 10% of HPE cases, “Medium” for genes mutated between 10% and 1%, and “Low” for genes mutated in less than 1% of HPE cases. Mutation frequencies were calculated based on our local HPE cohort (>1000 cases). Mainly inherited: inherited mutations from a parent are predominant among the reported cases; inherited: All reported mutations are inherited from a parent; NA: inheritance information was not available. CNV or large indels encompassing whole genes are not included in this table.

Table 2. Digenic inheritance in human HPE cases

Mutations are given in proteic nomenclature, except the del18p11 which carries the TGIF1 gene off.

Tables 3. Phenotypic description of families F1 and F2 members.

(a) Phenotypic description of family F1 members. The head circumferences of individuals harboring microcephaly are given in standard deviation (SD). Facial description of fetus III3 was not available (NA). The dash means that the phenotype is normal. (b) Phenotypic description of family F2 members. M=Male, and F=Female.

Table 4. Characteristics of *DISP1* and *SHH* mutations found in F1 and F2 families

Mutations reported in F1 family (*DISP1* p.Met1096Thr; *SHH* p.Pro347Gln) and in F2 family (*DISP1* p.Asn363Asp; *DISP1* p.Glu553Lys) were annotated using ANNOVAR. Minor alleles frequencies were extracted from dbSNP build 138, in the Exome Sequencing Project containing sequencing data from 6500 exomes (ESP6500), in the 1000 Genome Project release of 2014 (1000G), and in the Exome Annotation Consortium (ExAC) containing sequencing data from 60700 exomes. Bioinformatic predictions were given by 10 predictions tools (SIFT, PolyPhen-2 HDIV and HVAR, LRT, Mutation Taster, Mutation Assessor, FATHMM, Radial SVM, LR and CADD). The cumulated predictions were given here, D=Deleterious, P=Possibly Deleterious, T=Tolerated.

TABLES

Gene	Chromosomal Locus	Mutation frequency in nonsyndromic HPE	Percentage of Inherited mutations from a parent	Zygosity state	References
SHH	7q36	High (12%)	70%	Heterozygous	(5, 23, 45, 56-58)
ZIC2	13q32	Medium (9%)	30%	Heterozygous	(5, 45, 59-62)
SIX3	2p21	Medium (5%)	70%	Heterozygous	(5, 45, 63-67)
TGIF1	18p11.3	Medium (1,7%)	Mainly inherited	Heterozygous	(5, 68, 69)
PTCH1	9q22.3	Low	Mainly inherited	Heterozygous	(4, 70, 71)
TDGF1	3p23-p21	Low	NA	Heterozygous	(10)
GLI2	2q14	Low	Mainly inherited	Heterozygous	(70, 72, 73)
DISP1	1q42	Low	Mainly inherited	Heterozygous	(38, 39)
FGF8	10q24	Low	Mainly inherited	Heterozygous Homozygous	(11, 47)
FOXH1	8q24.3	Low	NA	Heterozygous	(74)
NODAL	10q22.1	Low	NA	Heterozygous	(75)
GAS1	9q21.33	Low	Mainly inherited	Heterozygous	(12)
DLL1	6q27	Low	Inherited	Heterozygous	(15)
CDON	11q24.2	Low	NA	Heterozygous	(13)
STIL	1p33	Low	Inherited	Heterozygous Homozygous	(8, 9, 76)

Table 1. Characteristics of genes and mutations implicated in holoprosencephaly.

Gene	Mutation	References
SHH	p.Gly290Asp	(23)
ZIC2	p.Ala461_Ala470dup	
SHH	p.Pro424Ala	(23)
TGIF1	del18p11	
SHH	p.del378_380	(23)
TGIF1	p.Thr151Ala	
GLI2	p.Arg151Gly	(70)
PTCH1	p.Thr328Ala	
SIX3	p.Ala93Asp	(4)
PTCH1	p.Ala393Thr	
SIX3	p.Ala284Pro	(77)
ZIC2	p.Trp304Arg	
SHH	p.Leu218Pro	(12)
GAS1	p.Asp270Tyr	
SHH	p.Cys363Tyr	(12)
GAS1	p.Asp288Gly	
SHH	p.Pro347Gln	In this report
DISP1	p.Met1096Thr	

Table 2. Digenic inheritance in human HPE cases

(a)

	Sex	Age	Brain MRI	Face
I1	M	81	Microcephaly (-5SD)	**-**
I2	F	79	**-**	**-**
II1	M	48	Microcephaly (-5SD)	-
II2	F	48	-	-
II3	M	46	Microcephaly (-4,5SD)	Hypotelorism
II5	M	42	-	-
II6	F	35	-	-
III1	M (fetus)	-	Semi-lobar HPE	Proboscis Macroglossy
III2	F (fetus)	-	Alobar HPE	Flat face hypotelorism Premaxillary agenesia Cleft lip/palate
III3	F (fetus)	-	Severe HPE	NA
III4	F	12	-	-
III5	F	11	-	-
III6	M	12	Microcephaly (-4SD)	Hypotelorism Cleft lip/palate
III7	M	11	Microcephaly (-3SD)	-
III8	F	9	-	-
III9	F	6	Microcephaly (-4SD)	-

(b)

	Sex	Age	Brain MRI	Face
I1	M	34	-	-
I2	F	33	-	-
II1	F	9	Lobar HPE Microcephaly (-2SD)	Flat face Short nose Hypotelorism Arched palate Cleft palate Microstomia Narrow palpebral fissures

Tables 3. Phenotypic description of families F1 and F2 members.

Gene	Nucleic acid change	Amino acid change	Minor Allele Frequencies				Bioinformatic predictions
			dbSNP	ESP6500	1000G	ExAC	
DISP1	c.T3287C	p.Met1096Thr	rs144673025	0,005	0,001	0,006	D:3 P:1 T:6
SHH	c.C1040A	p.Pro347Gln	-	0	0	0	D:9 P:1 T:0
DISP1	c.A1087G	p.Asn363Asp	-	0	0	0	D:7 P:2 T:1
DISP1	c.G1657A	p.Glu553Lys	-	0	0	0	D:5 P:1 T:4

Table 4. Characteristics of *DISP1* and *SHH* mutations found in F1 and F2 families