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FREX Consortium; GoNL Consortium; Kim, Artem; Savary, Clara; Dubourg, Christele; Carre, Wilfrid; Mouden, Charlotte; Guyodo, Helene; Le Douce, Jerome; Pasquier, Laurent

Published in:
Brain

DOI:
10.1093/brain/awy290

doi:10.1093/brain/awy290 BRAIN 2019: 142; 35–49 | 35

BRAIN
A JOURNAL OF NEUROLOGY

Integrated clinical and omics approach to rare diseases: novel genes and oligogenic inheritance in holoprosencephaly

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Holoprosencephaly is a pathology of forebrain development characterized by high phenotypic heterogeneity. The disease presents with various clinical manifestations at the cerebral or facial levels. Several genes have been implicated in holoprosencephaly but its genetic basis remains unclear: different transmission patterns have been described including autosomal dominant, recessive and digenic inheritance. Conventional molecular testing approaches result in a very low diagnostic yield and most cases remain unsolved. In our study, we address the possibility that genetically unsolved cases of holoprosencephaly present an oligogenic origin and result from combined inherited mutations in several genes. Twenty-six unrelated families, for whom no genetic cause of holoprosencephaly could be identified in clinical settings [whole exome sequencing and comparative genomic hybridization (CGH)-array analyses], were reanalysed under the hypothesis of oligogenic inheritance. Standard variant analysis was improved with a gene prioritization strategy based on clinical ontologies and gene co-expression networks. Clinical phenotyping and exploration of cross-species similarities were further performed on a family-by-family basis. Statistical validation was performed on 248 ancestrally similar control trios provided by the Genome of the Netherlands project and on 574 ancestrally matched controls provided by the French Exome Project. Variants of clinical interest were identified in 180 genes significantly associated with key pathways of forebrain development including sonic hedgehog (*SHH*) and primary cilia. Oligogenic events were observed in 10 families and involved both known and novel holoprosencephaly genes including recurrently mutated *FAT1*, *NDST1*, *COL2A1* and *SCUBE2*. The incidence of oligogenic combinations was significantly higher in holoprosencephaly patients compared to two control populations ($P < 10^{-9}$). We also show that depending on the affected genes, patients present with particular clinical features. This study reports novel disease genes and supports oligogenicity as clinically relevant model in holoprosencephaly. It also highlights key roles of SHH signalling and primary cilia in forebrain development. We hypothesize that distinction between different clinical manifestations of holoprosencephaly lies in the degree of overall functional impact on SHH signalling. Finally, we underline that integrating clinical phenotyping in genetic studies is a powerful tool to specify the clinical relevance of certain mutations.

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Received June 12, 2018. Revised August 21, 2018. Accepted September 28, 2018. Advance Access publication November 30, 2018

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Keywords: exome; holoprosencephaly; oligogenic inheritance; sonic hedgehog; primary cilia

Abbreviations: GoNL = Genome of the Netherlands; HPE = holoprosencephaly; WES = whole exome sequencing

Introduction

Holoprosencephaly (HPE1, OMIM #236100) is a severe developmental defect resulting from incomplete forebrain cleavage. The disease is characterized by incomplete separation of cerebral hemispheres with several anatomical classes ranging from microforms to alobar HPE. Affected individuals present with typical craniofacial midline defects of varying severity including proboscis, cleft lip and palate, ocular hypotelorism and solitary median incisor. HPE occurs in about 1 in 10 000 to 20 000 live births worldwide (Mercier *et al.*, 2011).

The genetic basis of HPE remains unclear and different transmission patterns have been described including autosomal dominant, recessive and digenic inheritance (Dubourg *et al.*, 2018). Most mutations associated with HPE display incomplete penetrance and variable expressivity, i.e. close relatives carrying the same pathogenic variant can be asymptomatic or present distinct HPE-spectrum anomalies (Mercier *et al.*, 2011). Sonic hedgehog (*SHH*) was the first discovered gene implicated in HPE (Roessler *et al.*, 1996) and its variants remain the most common cause of non-chromosomal HPE (Dubourg *et al.*, 2018). In 2011, molecular screening of 645 HPE probands revealed that mutations in the *SHH, ZIC2, SIX3* and *TGIF1* genes were the most frequent ones and collectively accounted for 25% of cases (Mercier *et al.*, 2011). The following studies reported that *GLI2* might also be considered as a major HPE gene in terms of frequency (Dubourg *et al.*, 2016), although variants in *GLI2* rarely result in classic HPE but instead cause a distinct phenotype that includes pituitary insufficiency and subtle facial features (Bear *et al.*, 2014). Pathogenic variants in *FGF8, FGFR1, DISP1*, and *DLL1* were also found in ~7% of HPE cases (Dupé *et al.*, 2011; Dubourg *et al.*, 2016). The other HPE genes reported so far are *TDGF1, FOXH1, TGIF1, CDON, NODAL, GAS1, STIL* and *SUFU* whose frequency is not established due to the small number of reported cases (Mouden *et al.*, 2015, 2016; Dubourg *et al.*, 2018; Kruszka *et al.*, 2018).

Clinical genetic testing of HPE has improved, but ~70% of familial cases remain without a clear molecular diagnosis. Most of known HPE genes belong to the SHH pathway, which represents the primary pathway implicated in the disease (Mercier *et al.*, 2013; Dubourg *et al.*, 2016; Kruszka *et al.*, 2018). Therefore, defective SHH-related processes are likely to be substantially involved in HPE.

Whole-exome sequencing (WES) has been successful for Mendelian disease-gene discovery and differential diagnosis (Bamshad *et al.*, 2011). WES analysis uses filtering approaches for candidate variant prioritization combined with comprehensive clinical evaluation. A variety of additional strategies has been developed to further improve the performance of WES in clinical settings. Collaborative platforms such as Matchmaker Exchange (Philippakis *et al.*, 2015) are used to search for recurrence in patients affected by similar phenotypes. Integrative variant-prioritization algorithms such as the Exomiser suite (Smedley *et al.*, 2015) combine WES with different phenotype-driven approaches (based on clinical data and cross-species phenotype comparisons) and analysis of protein interactome data. As useful as they are, these strategies are limited: collaborative platforms are not efficient in case of very rare genetic diseases while pipelines such as Exomiser are not designed to study non-Mendelian disorders. Studying HPE faces these two challenges: (i) HPE live-born infants are excessively rare; and (ii) although HPE is considered a Mendelian disorder, the wide range of severity must necessitate strong modifying factors such that a single pathogenic variant may be neither necessary nor sufficient for pathogenesis.

Recent studies have highlighted that non-Mendelian disease phenotypes could present an oligogenic aetiology and result from accumulation of inherited low-penetrance variants in multiple genes (Li *et al.*, 2017). However, such events are likely overlooked in clinical genetic studies if variants are inherited from a clinically unaffected parent.

In this study, we address the additional yield that can be obtained for HPE patients who underwent medical WES evaluation in clinical settings that failed to establish a molecular diagnosis. Given the wide clinical spectrum of the disease, as well as incomplete penetrance and variable expressivity of HPE mutations, we raised the possibility that the low diagnostic yield is partly due to the complex aetiology of HPE and hypothesized that a part of unsolved HPE cases results from oligogenic events, i.e. accumulation of several rare hypomorphic variants in distinct, functionally connected genes.

Our study involved patients for whom no disease aetiology could be determined by conventional diagnostic approaches. Similarly to previous WES studies (Lee *et al.*, 2014; Stark *et al.*, 2017), we used clinically-driven prioritization approach to identify genes associated with specific clinical features as reported in gene-phenotype reference databases and mouse models. Complementarily, we developed and used a prioritization strategy based on gene co-expression networks of the developing human brain to select genes with spatio-temporal expression patterns compatible with those of known HPE genes. Finally, we used in-depth clinical phenotyping together with cross-species similarities to further strengthen the evidence of causality.

This study highlights novel HPE genes and identifies new disease-related pathways including the primary cilia pathway. Our findings also illustrate the high degree of oligogenicity of HPE and suggest that the disease requires a joint effect of multiple hypomorphic mutations.

Materials and methods

Patient selection and preliminary genetic analyses

Study protocol was approved by the Ethics Committee of Rennes Hospital. Patients diagnosed with HPE and relatives were recruited using the clinical database of Holoprosencephaly Reference Center of Rennes Hospital. Study participation involved informed written consent, availability of clinical data, and either DNA or peripheral blood sample.

The main selection criterion for this study was the absence of clear genetic cause of HPE after conventional diagnostic procedures. As part of routine diagnosis, all patients were scanned for rare damaging mutations by targeted HPE gene-panel sequencing (Dubourg *et al.*, 2016) and for copy number variants (CNVs) using comparative genomic hybridization (CGH)-array and multiplex ligation-dependent probe amplification (MLPA). Patients for whom no genetic cause of HPE (i.e. a fully-penetrant causal mutation in known HPE gene or a chromosomic aberration/copy number variant explaining the pathology) could be established, underwent trio-based WES for further analysis. WES was performed using standard procedures as previously described (Mouden *et al.*, 2015, 2016). The scheme for variant classification followed the American College of Medical Genetics and Genomics association (ACMG) guidelines (Richards *et al.*, 2015) and included a hypothesis-free analysis of all *de novo* and homozygous variants on a family-by-family basis. Patients for whom no such variants of clinical interest had been detected were considered eligible for the hypothesis of oligogenic inheritance and included in this study.

Variant selection under oligogenic hypothesis

As discussed in previous studies, ACMG guidelines are useful in identifying variants with strong effect on phenotype but are unhelpful in case of modifier variants (Hong *et al.*, 2017). Therefore, the ACMG classification was not taken into account for variant selection dedicated to the analysis of oligogenic events. WES trio data were reanalysed using more permissive settings (filtering protocols used in this study are described in the Supplementary material). The exome analysis was complemented with two gene prioritization strategies based on available clinical knowledge and co-expression networks.

Clinically-driven approach

We established two clinician-generated lists of relevant phenotypes reminiscent of HPE in human and mouse models, respectively (Supplementary Table 3). Genes associated with the phenotypes of interest were identified with publicly available clinical resources and associated ontologies. Human gene-phenotype associations were extracted from relevant databases (Supplementary Fig. 1) using R package *VarFromPDB* (https://github.com/cran/VarfromPDB). The Mouse Genome Informatics (MGI) (Smith *et al.*, 2018) database and a homemade workflow were used to retrieve genes associated with any of the corresponding phenotypes in mouse mutants. Human and mouse results were combined and redundancy was removed to establish a list of clinically-driven candidate genes associated with HPE-related anomalies (Supplementary Table 4).

Identification of HPE-related genes by weighted gene co-expression network analysis

We used weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) on the RNA-Seq data from the Human Development Biology Resource (HDBR) (Lindsay *et al.*, 2016) to identify genes sharing highly similar expression patterns with four classical genes associated with HPE (*SHH, SIX3, ZIC2* and *TGIF1*) during cerebral development. Data from samples corresponding to forebrain, cerebral cortex, diencephalon, telencephalon and temporal lobe structures taken between the fourth and 10th post-conception weeks were selected (Supplementary Fig. 9). RNA-seq data were analysed with the iRAP pipeline (https://github.com/nunofonseca/irap). We used R package WGCNA to construct co-expression networks and identify modules of co-expressed genes. The detailed protocols for WGCNA analysis are described in the Supplementary material. The Topological Overlap Matrix (TOM) matrix was used to establish a list of transcriptome-driven candidate genes sharing highly similar expression profiles with *SHH, ZIC2, SIX3* and *TGIF1* (Supplementary Table 5).

Integration and identification of oligogenic events

The two gene prioritization schemes were combined with the WES results to identify a restricted list of rare variations located in genes identified by either the transcriptomic or the clinical prioritization approach (Fig. 1). Further analyses of the candidate variants were performed on a family-by-family basis. Oligogenic events were defined as combinations of candidate variants in $\geqslant 2$ genes co-segregating with disease, i.e. unique to

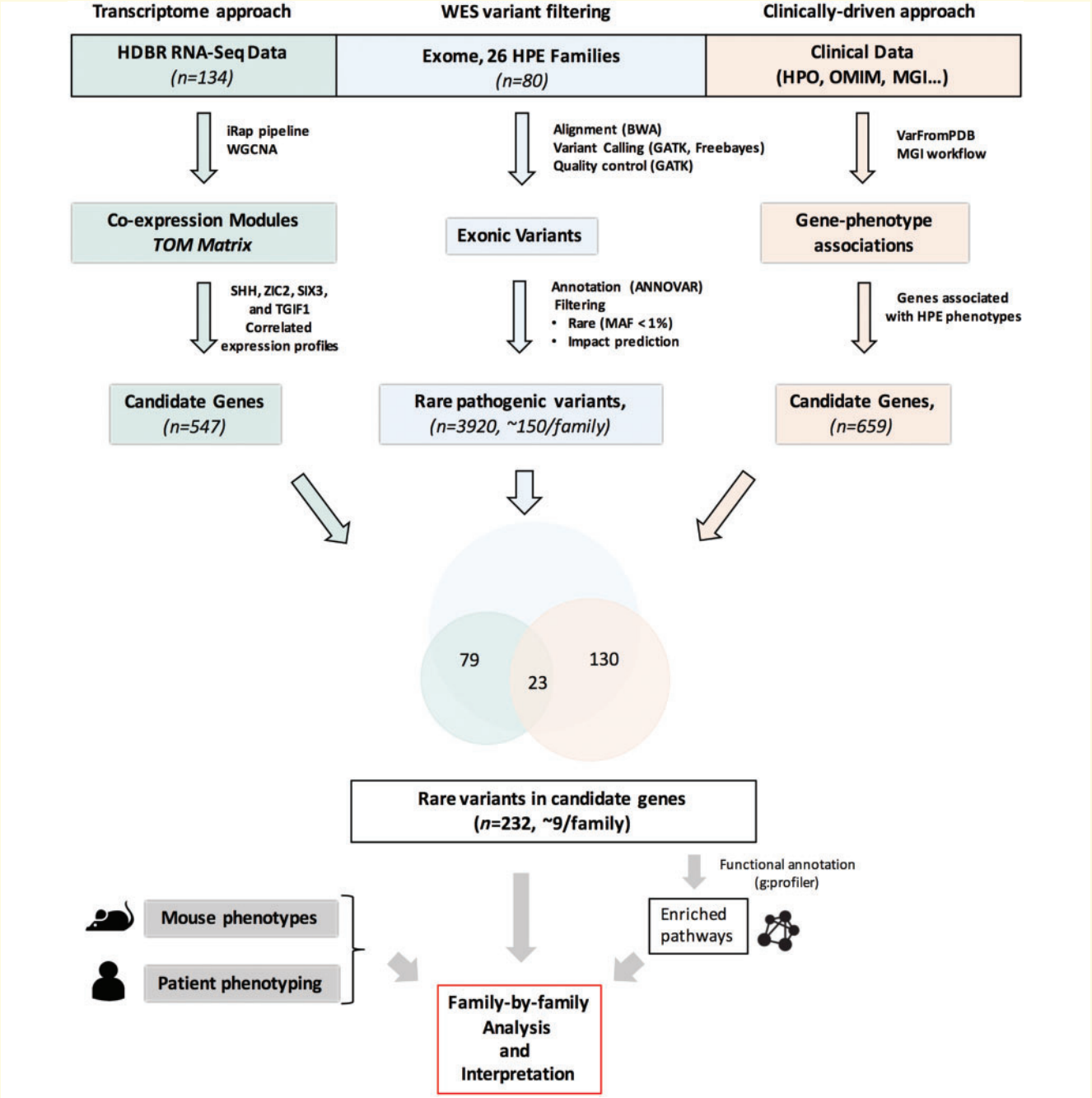

Figure 1 Flow chart illustrating the prioritization strategy. Classical WES analysis was performed (blue) and combined with two prioritization approaches: (i) based on gene co-expression networks (green); and (ii) based on clinical knowledge (salmon). Details of the pipeline are also provided in the Supplementary material. Variant overlaps were selected and further analysed by functional annotation analysis and on a family-by-family basis, by integrating a comprehensive clinical phenotyping of patients and exploration of cross-species similarities.

the affected individuals of each family. Variants could be either inherited from the parents—at least one each from the mother and the father—or occur *de novo* in the affected child.

To evaluate the impact of candidate genes further, we performed deep clinical phenotyping to characterize similarities between unrelated patients and/or published knockout mice. Special attention was given to genes harbouring distinct rare variants in at least two affected patients with striking phenotypic overlap. Phenotypic overlaps between patients and mouse mutants deficient for the corresponding candidate genes were also examined. The most interesting oligogenic combinations of rare deleterious variants in the affected children were finally discussed during multidisciplinary meetings.

To determine significantly enriched biological processes and pathways, functional annotation was performed by *g:profiler* (http://biit.cs.ut.ee/gprofiler) and Bonferroni adjusted *P*-value were considered significant below a value of 0.05 (KEGG, REACTOME and Gene Ontology Biological Processes).

Control cohorts and validation

To test whether the identified oligogenic combinations were specific to the HPE cohort, we used SNV and INDELS data from 248 healthy trios (744 individuals) provided by Genome of the Netherlands (GoNL) sequencing project as a control cohort (Genome of the Netherlands Consortium, 2014). Additional control cohort consisting of 574 unrelated French individuals was provided by the French Exome Project (FREX).

We applied the same variant filtering approach and the same strategy for selection of oligogenic events. Proportion of families and/or individuals presenting oligogenic events were then compared between HPE cohort and the control cohorts. *P*-values were calculated using two-sided Fisher's exact test (*fisher.test* function in R, version 3.4.2).

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Clinical findings

We assembled a cohort of 26 families representing a total of 80 individuals including 29 affected children diagnosed with lobar ($n = 3$), semilobar ($n = 11$), alobar ($n = 13$) or microform HPE ($n = 2$) (Table 1). Common HPE clinical manifestations were observed among the probands and included cleft lip and palate (38%), hypotelorism (34%), microcephaly (31%) and arhinencephaly (31%). Ancestry analysis identified that 24 families were of European descent and two of South East Asia and African descent (Supplementary Fig. 10). Eight parents presented minor signs of midline facial anomalies and three parents were diagnosed with HPE microforms.

The initial targeted sequencing had identified point mutations in known HPE genes in 13 families and a full heterozygous deletion of *SIX3* gene had been detected by CGH-array in one family (Fig. 2 and Supplementary Fig. 8). All anomalies were later confirmed by WES analysis. They were inherited from asymptomatic or mildly affected parents and were considered as insufficient to fully explain the pathogenesis of HPE, suggesting that the presence of additional risk factors was required for the disease to occur.

HPE variants overview and identification of disease-related pathways

Combined clinically- and transcriptome-driven analysis of the exome data identified a total of 232 rare candidate variants in 180 genes (Fig. 1 and Supplementary Table 6). All variants presented a minor allele frequency below 1% and were predicted to be highly deleterious to protein function (Supplementary material). One hundred and fifty-three variants concerned genes associated with HPE phenotypes among which 32 were located in genes reported to induce HPE-like phenotypes in mutant mice (Supplementary Table 8). One hundred and two variants were located in genes sharing expression profiles highly similar to those of

Table 1 Clinical description of 26 HPE families

Category and feature	*n*	%
Proband sex		
Male	6	21
Female	20	69
Unknown	3	10
Total	**29**	100
Clinical phenotype of the parents		
Unaffected	40	78
Minor sign	8	16
Hypotelorism	4	8
Incomplete iris	1	2
Epicanthus	1	2
Narrow palate	1	2
Nasal anomaly	1	2
HPE microform	3	6
Total	**51**	100
Clinical characteristics of the probands		
HPE	29	100
Lobar	3	10
Semilobar	11	38
Alobar	13	45
Microform	2	7
Cleft lip/palate	11	38
Hypotelorism	10	34
Microcephaly	9	31
Arhinencephaly	9	31
Agenesis of corpus callosum	7	24
Flat head (plagiocephaly)	6	21
Thalami Fusion	6	21
Ventricles Fusion	6	21
Premaxilliary agenesia	5	17
Fusion frontal lobes	4	14
Flat nose	4	14
Proboscis	3	10
Cyclopia	2	7
Total	**29**	100
Families with mutations in HPE genes		
SHH	4	15.4
ZIC2	1	3.8
SIX3	5*	19.2
TGIF1	2	7.7
PTCH1	1	3.3
ZIC2/GLI2	1	3.8
No mutation	12	46.2
Total	**26**	100.0
Family ethnicity		
European	21	81
African	1	4
South Asian	1	4
Admix	3	12
Total	26	100.0

*For *SIX3*, point mutations were found in four families (targeted sequencing) and a heterozygous deletion was detected by CGH-array in one family.

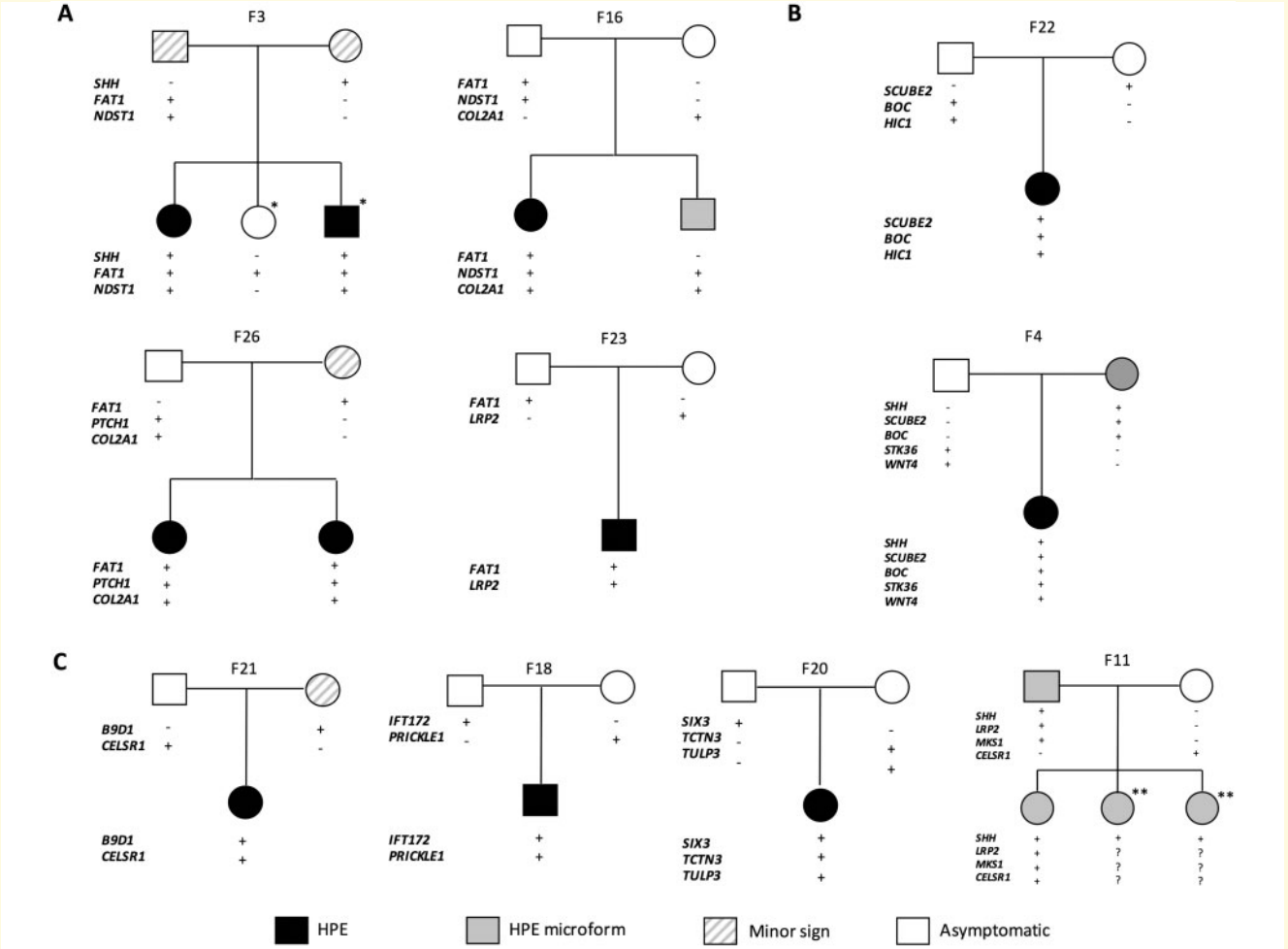

Figure 2 Oligogenic events reported in this study. Candidate genes are listed for each family. Individuals carrying or not carrying the variants are identified by the plus or minus sign symbols, respectively. Variant information is available in Tables 2, 3 and Supplementary Table 6. (**A**) Oligogenic events involving *FAT1*. (**B**) Oligogenic events involving variants in *SCUBE2* and *BOC*. (**C**) Oligogenic events involving mutations in genes related to the primary cilium. *Not available for WES, clinical phenotyping and Sanger sequencing of *SHH, FAT1* and *NDST1* were performed. **Samples not available, Sanger sequencing of *SHH* was performed in the referring laboratory.

HPE genes. Overlap between phenotype and gene co-expression network analysis contains 23 variants including 14 previously described mutations in known HPE genes (*SHH, ZIC2, SIX3, GLI2, TGIF1* and *PTCH1*).

Consistent with known disease aetiology, functional profiling of the 180 genes revealed a significant enrichment for biological processes implicated in forebrain development (Supplementary Table 7) including Sonic Hedgehog signalling pathway (REAC:5358351, P-value = 2.79×10^{-5}; KEGG:04340, P-value = 10^{-4}), Primary Cilia (REAC:5617833, P-value = 10^{-6}; GO:0060271, P-value = 2×10^{-6}) and Wnt/Planar Cell Polarity (PCP) signalling pathway (GO:0016055, P-value = 2×10^{-5}). The SHH pathway is the primary pathway implicated in HPE and the primary cilium is required for the transduction of SHH signalling (Gorivodsky *et al.*, 2009; Murdoch and Copp, 2010) while components of Wnt/PCP pathway regulate both SHH signalling and primary cilia (Goetz *et al.*, 2009; Murdoch and Copp, 2010).

In-depth analyses highlighted 10 families with oligogenic events (Fig. 2) clustered among 19 genes (Tables 2 and 3) that functionally relate to disease-relevant pathways (Fig. 3). These combinations of variants were unique to the affected probands. The main findings are presented below and full reports are available in the Supplementary material.

Recurrent oligogenic events involving *FAT1*

Four different families, i.e. 15% of the 26 families studied here, presented oligogenic events involving *FAT1* in combination with rare variants in known HPE genes (*SHH, PTCH1)*, as well as in *NDST1, COL2A1* and *LRP2*

Table 2 Comparison of clinical features in the studied families

Phenotype	HPO	Family 3					Family 16				Family 23			Family 26				Family 22			Family 4			Family 21			Family 18			Family 11			Family 20		
		F	M	P	Fo	S	F	M	P	P2	F	M	P	F	M	P	Fo	F	M	P	F	M	P	F	M	P	F	M	P	F	M	P	F	M	P
Alobar HPE	HP:0006988												+			+	+						+			+			+						+
Semilobar HPE	HP:0002507			+	+				+								–			+															
Microform HPE	-									+							–					+									+			+		
Proboscis	HP:0012806			+	–												–									+									
Abnormal nose morphology	HP:0005105			+	+				+	+						+	–			+			+			+			+						
Monorhinia	-																			+			+												
Mandibular anomalies	HP:0000277			+	–				+	+							–									+									
Abnormality of the outer ear	HP:0000356			+	–				+	+			+			+	–									+									+
Arhinencephaly	-			+	–												–						+			+									+
Abnormal olfactory bulb	HP:0040327			+	–				+							+	–												+						
Thalami fusion	HP:0010664			+	–				+							+	–									+			+						+
Agenesis corpus callosum	HP:0007370			+	–				+	+							–									+			+						+
Microcephaly	HP:0000252			+	–				+								–									+									+
Eye defects		+	+	+	–												–			+			+		+	+			+			+			+
Hypotelorism	HP:0000601	+		+	–												–			+			+						+						
Cyclopia	HP:0009914				–												–									+									
Epicanthus	HP:0000286		+		–												–																		
Aplasia/hypoplasia of iris	HP:0008053																–							+											
Falx cerebri abnormalities	HP:0010653				–				+				+				–									+									
Bilateral cleft lip and palate	HP:0002744			+	–								+				–						+			+			+			+			
IUGR	HP:0001511				–				+							+	–																		
Cebocephaly	-				–												–			+			+												
Turricephaly	HP:0000262			+	–												–									+									
Polydactyly	HP:0010442				–												–												+						+
Single umbilical artery	HP:0001195															+	–			+															
Narrow palate	HP:0000189														+											+									
Single median incisor	HP:0006315																													+					

Occurrences of phenotypes are marked with a plus symbol for each individual. A dash is used when no observation was possible on foetuses. F = father; Fo = foetus; IUGR = intrauterine growth retardation; P = proband; M = mother; S = sister.

Table 3 Comparison of genetic features in the studied families

Gene	Family 3						Family 16					Family 23				Family 26					Family 22			
	Allele	F	M	P	F	S	Allele	F	M	P	P2	Allele	F	M	P	Allele	F	M	P	F	Allele	F	M	P
SHH[a,b]	Asp171His		+	+	+																			
FAT1[b]	Tyr1770Cys	+		+	+	+	Val3459Met	+		+		Gly855Arg	+		+	Val3629Leu		+	+	+				
NDST1[b]	Arg80His	+		+	+		Arg132Cys	+		+	+													
COL2A1							Arg68His		+	+	+					Pro365Ser	+		+	+				
PTCH1[a,b]																Pro1211Ser	+		+	+				
LRP2[b]												Asn3205Asp		+	+									
BOC[a,b]																					Ala311Val	+		+
SCUBE2																					Arg525*		+	+
HIC1[b]																					Trp511Cys	+		+
STK36																								
WNT4																								
B9D1[b]																								
CELSR1																								
MKS1[b]																								
IFT172[b]																								
PRICKLE1																								
SIX3[a,b]																								
TCTN3																								
TULP3																								

	Family 4				Family 21				Family 18				Family 11				Family 20			
	Allele	F	M	P	Allele	F	M	P	Allele	F	M	P	Allele	F	M	P	Allele	F	M	P
SHH[a,b]	Phe241Val		+	+									Pro347Arg	+		+				
FAT1																				
NDST1																				
COL2A1																				
PTCH1[a,b]																				
LRP2													Glu3089Lys	+		+				
BOC[a,b]	Val861Ile		+	+																
SCUBE2	Thr285Met		+	+																
HIC1																				
STK36	Arg497Gly	+		+																
WNT4	Val204Met	+		+																
B9D1					Arg141Gln		+	+												
CELSR1					Arg954Cys	+		+					Tyr2820Cys		+	+				
MKS1													Gly255Arg	+		+				
IFT172									Ala961Val	+		+								
PRICKLE1									Ser739Phe		+	+								
SIX3[a,b]																	Del	+		+
TCTN3																	Asn512Ser		+	+
TULP3																	Ser437Thr		+	+

Heterozygous variants are marked with a plus symbol. F = father; Fo = foetus; IUGR = intrauterine growth retardation; P = proband; M = mother; S = sister.

[a]Are known HPE disease genes in humans.

[b]Mouse mutants exhibiting HPE exist.

genes (Fig. 2A). FAT1 is a protocadherin and its knockdown in mice causes severe midline defects including HPE (Ciani *et al.*, 2003); in *Drosophilia* it has been shown to regulate the PCP pathway (Rock *et al.*, 2005). *LRP2*, *NDST1* and *COL2A1* are all functionally relevant to the SHH pathway (Fig. 3): *NDST1* and *COL2A1* mice mutants exhibit HPE phenotype and reduced SHH signalling in the forebrain (Grobe *et al.*, 2005; Leung *et al.*, 2010), while *LRP2* acts as an auxiliary receptor of SHH during forebrain development and its inactivation in mouse similarly leads to HPE phenotype (Christ *et al.*, 2012).

Oligogenic events involved the following combinations: *SHH/FAT1/NDST1* (Family F3), *FAT1/NDST1/COL2A1* (Family F16), *FAT1/COL2A1/PTCH1* (Family F26) and *FAT1/LRP2* (Family F23) (Fig. 2A, Tables 2 and 3). Details are provided in the Supplementary material, Case report 1.

In Family F3, Sanger sequencing of additional family members revealed that the *SHH/FAT1/NDST1* combination was unique to the affected individuals (Fig. 2A). For Family F16, only the foetus carrying the *FAT1/NDST1/COL2A1* combination was affected by semilobar HPE, while the sibling carrying *NDST1/COL2A1* variants presented only a microform (Fig. 2A). These observations are fully consistent with the oligogenic inheritance model where accumulation of multiple variants in genes associated to HPE phenotypes and/or HPE-related molecular pathways is required.

Recurrent oligogenic events involving *SCUBE2/BOC* implicated in SHH signalling

Two families presented oligogenic events implicating combined variants in the *BOC* and *SCUBE2* genes (Fig. 2B, Tables 2 and 3). BOC is an auxiliary receptor of SHH and was recently reported as an HPE modifier in humans (Hong

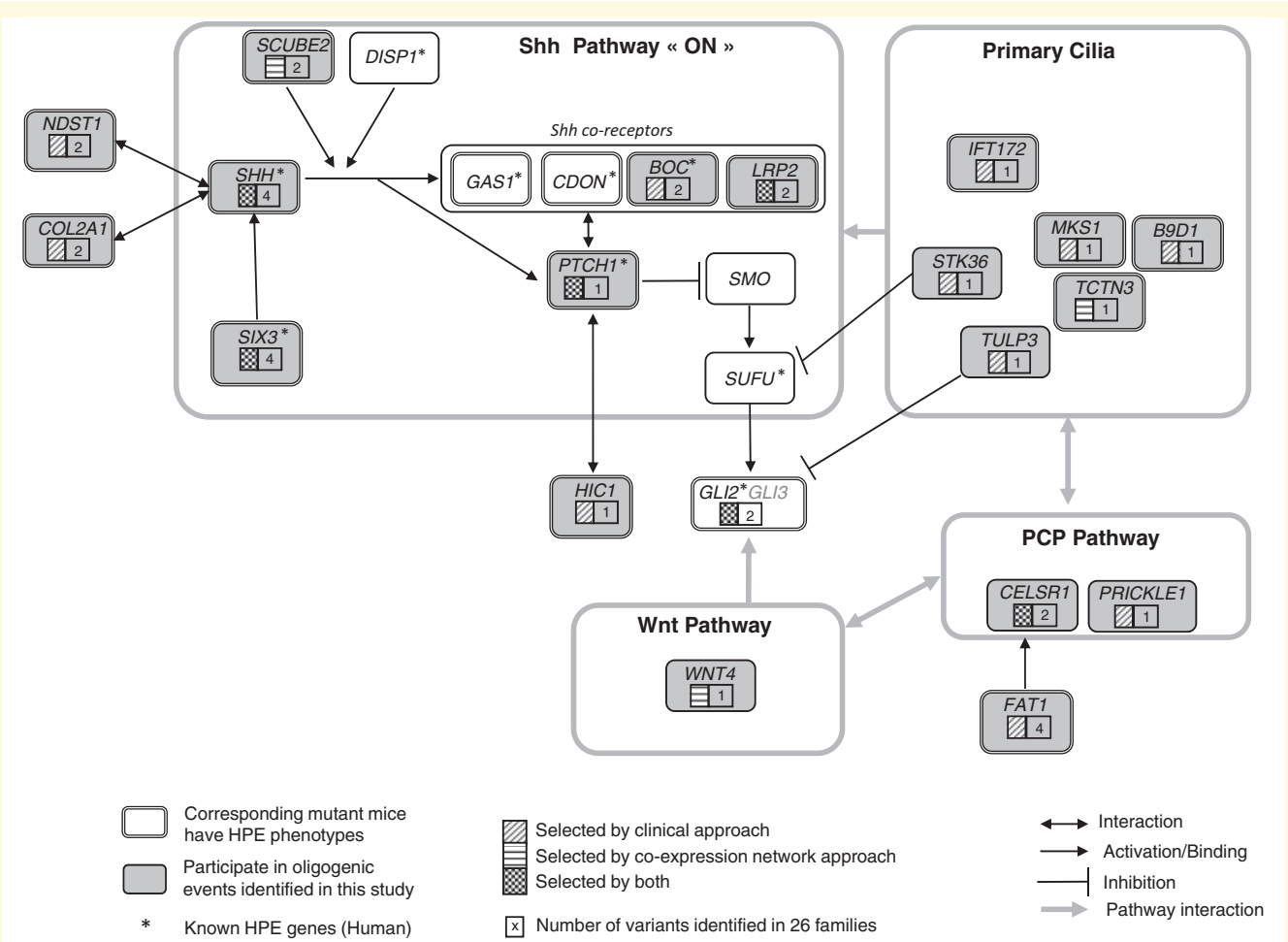

Figure 3 Implication of the candidate genes in the signalling pathways involved in HPE. Key affected pathways and genes are presented. Under each gene name, the selection methods (clinical or co-expression networks approach or both) is shown on the *left* and the number of variants for each gene is shown on the *right*. Genes known in HPE are marked with an asterisk, and genes for which corresponding mutant mice have HPE phenotypes are surrounded by a double line. The genes implicated in oligogenic events in the study are indicated with a grey background.

et al., 2017). *SCUBE2* shares a highly similar expression pattern with *SHH* and *SIX3* and is implicated in the release of *SHH* from the secreting cell (Jakobs *et al.*, 2014). In Family F4, a combination of *SCUBE2/BOC* variants was associated with additional variants in *SHH, STK36* (see below) and *WNT4*, a member of the Wnt pathway, implicated in regulation of SHH signalling (Murdoch and Copp, 2010). In Family F22, the *SCUBE2* variant results in a premature stop codon at position 525 (Supplementary Fig. 7), which results in truncation of its CUB domain and is predicted to directly affect its SHH-related activity (Jakobs *et al.*, 2014). This family presented an additional candidate variant in *HIC1*, which genetically interacts with *PTCH1* (Briggs *et al.*, 2008). Mice deficient for *HIC1* exhibit craniofacial defects including HPE (Carter, 2000).

The reported variant combinations were observed exclusively in the affected probands and were absent in asymptomatic individuals. Altogether, these results reveal recurrent mutations in *SCUBE2/BOC* and further strengthen the oligogenic inheritance model of HPE.

Implication of primary cilium in HPE

Remarkably, five families presented candidate variants in genes related to the primary cilium: *STK36, IFT172, B9D1, MKS1, TCTN3* and *TULP3* (Fig. 2C). Ciliary proteins are known to play essential roles in the transduction of SHH signalling downstream of PTCH1 during forebrain development (Goetz *et al.*, 2009; Murdoch and Copp, 2010).

STK36, also known as 'fused', is a ciliary protein implicated in SHH signalling and associated to craniofacial phenotypes (Goetz *et al.*, 2009; Murdoch and Copp, 2010). *IFT172* codes for a core component of intraflagellar transport complex IFT-B required for ciliogenesis and regulation of SHH signal transduction. Moreover, *Ift172*$^{-/-}$ mice exhibit reduced expression of *Shh* in the ventral forebrain and severe craniofacial malformations including HPE (Gorivodsky *et al.*, 2009). *B9D1, MKS1* and *TCTN3* are all members of the transition zone protein complex implicated in regulation of ciliogenesis (Garcia-Gonzalo *et al.*, 2011). The disruption of *B9d1* and *Mks1* in mouse models causes craniofacial defects that include HPE (Dowdle *et al.*, 2011; Wheway *et al.*, 2013). Although no mouse model is available for *TCTN3*, its expression profile is highly similar to that of *SHH* and disruption of its protein complex partners (*TCTN1, TCTN2, CC2D2A, MKS1, B9D1*) leads to HPE in mouse (Dowdle *et al.*, 2011; Garcia-Gonzalo *et al.*, 2011; Wheway *et al.*, 2013). Moreover, *TCTN3* was shown to be necessary for the transduction of SHH signal and *TCTN3* mutations were found in patients affected by ciliopathies (Thomas *et al.*, 2012). Finally, *TULP3* is a critical repressor of *Shh* signalling in mice and is associated with various craniofacial defects (Murdoch and Copp, 2010).

Additional variants observed in these families include a heterozygous deletion of *SIX3*, missense mutations in *SHH, SCUBE2, BOC* and *LRP2* (described above) as well as two genes implicated in PCP pathway (Fig. 3): *CELSR1* (two families) and *PRICKLE1*, both associated with craniofacial defects in mouse mutants (Fig. 2C) (Goetz *et al.*, 2009; Murdoch and Copp, 2010; Yang *et al.*, 2014). Similar to previously described cases, the oligogenic events were present exclusively in the affected children.

Given the essential role of the primary cilium in SHH signal transduction, these observations strongly suggest that rare variants in ciliary genes contribute to the disease onset in these families.

Correspondence between affected genes and secondary clinical features

To provide additional evidence, we performed an in-depth analysis of secondary clinical features associated with HPE in our patients. Deep clinical phenotyping identified clinical similarities between unrelated patients (Tables 2 and 3) as well as overlaps of secondary clinical features between patients and the corresponding mouse mutants.

Interestingly, the two patients with variants in ciliary genes (*IFT172/PRICKLE1* and *SIX3/TCTN3/TULP3*) both presented with polydactyly, a clinical feature commonly associated with ciliopathies (Goetz *et al.*, 2009). Importantly, the patient with the oligogenic combination *IFT172/PRICKLE1* presented with a large set of overlapping clinical features with the corresponding mouse mutants including polydactyly, cleft palate and eye defects (Gorivodsky *et al.*, 2009; Yang *et al.*, 2014).

Of note, the two unrelated patients having variants in *FAT1* and *NDST1* shared a large set of specific secondary clinical features, including mandibular and ear abnormalities. Intrauterine growth restriction was found exclusively in the two patients with *COL2A1* variants. The most severely affected child in Family F16 (*FAT1/NDST1/COL2A1*) presented a strong overlap with *NDST1*-null and *COL2A1*-null mutant mice (HPE, mandibular anomalies, absent olfactory bulb, abnormal nose morphology) (Grobe *et al.*, 2005; Leung *et al.*, 2010). Similarly, proboscis and eye defects were observed in both *FAT1/NDST1/SHH* patient and *FAT1*$^{-/-}$ mice (Ciani *et al.*, 2003).

Finally, the two unrelated *SCUBE2/BOC* cases in Families F4 and F22 presented with cebocephaly, a midline facial anomaly characterized by ocular hypotelorism and a single nostril, which was absent in all other patients. Consistently, *SCUBE2* is highly expressed in the nasal septum in mouse (Xavier and Cobourne, 2011), and cebocephaly was previously associated with *CDON*—another known HPE gene sharing highly similar functions and structure with *BOC* (Zhang *et al.*, 2006).

While these clinical features are not specific to HPE, the described overlaps provide additional support for disease implication of the presented candidate variants.

Statistical validations

The identified oligogenic events were clustered among 19 genes (Fig. 2, Tables 2 and 3). To assess the frequency of

Table 4 Statistical validations: Fisher's exact test analysis for oligogenic events

Comparison	HPE	GoNL	FREX	*P*-value		
				HPE versus GoNL	HPE versus FREX	GoNL versus FREX
Families with oligogenic events	10/26 (38%)	3/248 (1.2%)	NA	2.301×10^{-9}	NA	NA
Children harbouring rare deleterious variants in two or more candidate genes	13/29 (45%)	6/248 (2.4%)	NA	1.902×10^{-10}	NA	NA
All individuals harbouring rare deleterious variants in two or more candidate genes	21/80 (26%)	14/744 (1.8%)	16/574 (2.7%)	3.237×10^{-14}	1.521×10^{-11}	0.35

Oligogenic inheritance is defined as presence of combined rare deleterious variants in two or more genes, described in Table 3 and Fig. 2. The proportion of individuals harbouring combined rare deleterious variants in the identified genes is significantly higher in HPE cohort as compared to two control populations GoNL and FREX (Fisher's exact test).

healthy individuals presenting similar variant combinations in these genes, we applied the same family-by-family variant analysis to the 248 control trios provided by GoNL. This control cohort was chosen as 24/26 (92%) of the HPE families included in the study were of European descent (Supplementary Fig. 10).

The approach identified three families among controls presenting variant combinations satisfying the criteria that we established for the oligogenic events (gene, variant and parental inheritance). The three oligogenic events found in the control cohort were *FAT1/B9D1, SCUBE2/PTCH1* and *SCUBE2/LRP2/PTCH1/CELSR1* (Supplementary Table 9). Although one *SCUBE2* variant (p.Thr285Met) was found in both the HPE and the control cohort, none of the combinations found among controls corresponded to oligogenic events identified in the HPE cohort. The incidence of oligogenic events was significantly lower in the GoNL families (3/248, 1.2%) as compared to the HPE cohort (10/26, 38%) with a Fisher's exact test *P*-value of 2.301×10^{-9} (Table 4).

Three additional children of the GoNL cohort harboured combinations of rare deleterious variants in two or more candidate genes. However, in these cases, all variants were inherited from the same parent. Therefore, these combinations were not considered as oligogenic events similar to those of HPE patients. Nevertheless, even when taking into account these three additional cases, the proportion of children having variants in two or more candidate genes was significantly different between the HPE cohort (13/29, 45%) and the GoNL cohort (6/248, 2.4%) with a Fisher's exact test *P*-value of 1.902×10^{-10}.

Finally, 14 individuals of the GoNL cohort (parents and children combined) harboured rare deleterious variants in two or more genes. Without taking into account the relatedness between the GoNL individuals, the proportion of individuals having variants in two or more candidate genes remained significantly different between the HPE cohort (21/80, i.e. 26%) and the GoNL control cohort (14/744, 1.8%), as confirmed by Fisher's exact test (*P*-value = 3.237×10^{-14}).

To assess the frequency of control individuals presenting rare variant combinations in the identified candidate genes further (Fig. 2, Tables 2 and 3), we analysed a second control cohort. The FREX data were chosen as they consist of 574 unrelated French individuals ancestrally matching the HPE cohort.

Screening of the FREX cohort revealed that 16/574 individuals (i.e. 2.7%) harboured rare deleterious variants in two or more candidate genes. This proportion was statistically different from that observed in the HPE cohort (21/80, 26% versus 16/574, 2.7%; *P*-value = 1.521×10^{-11}, Fisher's exact test).

Additionally, the two control cohorts (GoNL and FREX) did not present statistically significant differences in terms of proportions of individuals having rare deleterious variants in two or more candidate genes: 14/744 (1.8%) for the GoNL cohort versus 16/574 (2.7%) for the FREX (*P*-value = 0.35, Fisher's exact test).

The analysis of the GoNL and FREX cohorts illustrates that the incidence of combined rare deleterious variants in the identified candidate genes is significantly higher in HPE patients as compared to a control population. All performed comparisons showed a statistically significant *P*-value between the cases and the controls (Table 4), thus providing evidence for oligogenicity as clinically relevant model in HPE.

Discussion

In this study, we addressed the relevance of oligogenic model for unsolved HPE cases. We provide evidence that the onset of HPE arises from the combined effects of hypomorphic variants in several genes belonging to critical biological pathways of brain development. To circumvent the limitations of classical WES analysis in complex rare disorders, we combined clinically-driven and co-expression network analyses with classical WES variant prioritization. This strategy was applied to 26 HPE families and allowed prioritization of 180 genes directly linked to the SHH signalling, cilium and Wnt/PCP pathways (Fig. 3). The analysis of oligogenic events in patients with HPE anomalies revealed 19 genes including 15 genes previously unreported in human HPE patients (Tables 2 and 3). All these genes

are either associated with HPE phenotypes in corresponding mouse models (such as *FAT1, NDST1*), present highly similar expression patterns with already known HPE genes in the developing brain (such as *SCUBE2, TCTN3*), or both. We observed co-occurrence of mutations in several gene pairs such as *FAT1/NDST1* and *SCUBE2/BOC*, which provides additional arguments towards their implication in HPE. The incidence of oligogenic combinations was significantly higher in HPE patients compared to the GoNL and FREX control populations. We additionally show that in-depth evaluation of secondary clinical features in patients with HPE anomalies and comparison to published mouse knockout models may provide additional arguments for the causality of candidate genes.

The main challenge in disease-gene discovery by WES is to identify disease-related variants among a large background of non-pathogenic polymorphisms (Bamshad *et al.*, 2011; MacArthur *et al.*, 2014). For example, the presented *FAT1* encodes a large protocadherin gene spanning over 139 kb in the human genome and presenting over 2000 missense variants with a minor allele frequency below 1% in the gnomAD database. Despite this high number of variations found in the general population, rare variants in *FAT1* were recently implicated in several genetic disorders including facioscapulohumeral dystrophy-like disease (Puppo *et al.*, 2015). Hence, correct interpretations and conclusions require extremely careful assessment of available biological and clinical knowledge.

To improve the pertinence of our study, we developed a strategy to restrict the potential candidates by targeting genes with biological and clinical arguments for their implication in the disease. Implication of a given gene in a disease is often supported by the similarity between the human pathology and the phenotype obtained in relevant animal models (MacArthur *et al.*, 2014). Accordingly, in this study, the main evidence of causality for candidate genes was that their disruption leads to clinically-defined HPE-related phenotypes in corresponding published mutant mouse models. Unlike other phenotypes, such as reduced body weight (Reed *et al.*, 2008), holoprosencephaly is a rare effect of gene knockout in mice as it is associated with $<1\%$ of knockout mice (as reported in the MGI database). Recent exome sequencing studies have applied similar phenotype-driven approaches to identify causal variants in monogenic disorders. Dedicated tools have been developed to that aim (Exomiser, Phive) (Smedley *et al.*, 2015) but none are designed for non-Mendelian traits involving hypomorphic variants with mild effects. We provide a method to specifically address such cases and show that further developments are necessary to improve the diagnosis of genetic disorders, especially by taking into account oligogenic inheritance. Inclusion of carefully defined mouse mutant phenotypes is of powerful value as certain phenotypes like HPE are very informative due to their rarity.

Prioritization tools can also include protein–protein interaction (PPI) network information, which improves performance in cases where candidate genes do not have an associated knockout mouse model. However, PPI-based prioritization is limited when disease investigation requires incorporation of tissue-specific data. The key process affected by HPE is the elaboration of the forebrain and its dorso-ventral patterning (Fernandes and Hébert, 2008). Deciphering the biological mechanisms involved in the early brain development is therefore necessary to provide relevant information to select disease-related genes. To incorporate tissue-specificity, we performed analysis using the RNA-Seq data of embryonic human brain at the earliest available developmental stages (from 4 to 17 post-conception weeks) as provided by the Human Development Biology Resource (Lindsay *et al.*, 2016). We defined relevant co-expression modules and selected candidate genes of which expression patterns follow those of known HPE genes. Further analysis showed that the resulting candidate genes, such as *SCUBE2* and *TCTN3*, are pertinent as they are equally implicated in the SHH pathway that is the primary HPE pathway (Thomas *et al.*, 2012; Jakobs *et al.*, 2014). Co-expression analysis provides additional insight into disease pathogenesis by establishing the first link between previously unrelated genes. A future challenge will be to generalize this approach, but such a task will face the necessity to incorporate disease relevant co-expression modules that need to be pre-computed.

Patients exhibiting HPE anomalies present enrichment of rare variants in genes related to the SHH pathway, as well as to the Wnt/PCP and primary cilia pathways, which were both shown to functionally interact with and regulate SHH pathway (Goetz *et al.*, 2009; Gorivodsky *et al.*, 2009; Murdoch and Copp, 2010; Wheway *et al.*, 2013). Accumulation of multiple rare variants in genes related to these pathways will likely disrupt the dorso-ventral gradient of the SHH morphogen (Fernandes and Hébert, 2008), leading to an incomplete cleavage of the forebrain and, ultimately, to HPE. In this model, distinction between different manifestations of HPE lies in the degree of overall functional impact on SHH signalling (Mercier *et al.*, 2013). Moreover, depending on the affected genes and pathways, HPE patients would present different secondary clinical features.

The observed overlapping secondary clinical features further support the causality of the reported variants for HPE. As hypomorphic mutations do not have the same impact as the complete inactivation of a gene in most cases, phenotypic overlaps may be challenging to detect and require expert assessment of clinical and biological data. For example, mice deficient in *NDST1* exhibit agnathia (Grobe *et al.*, 2005) (absence of the lower jaw) while unrelated patients presenting candidate variants in *NDST1* exhibit prognathia and retrognathia (abnormal positioning of the lower jaw), respectively. All three phenotypes are part of the same spectrum of mandibular anomalies. From a clinical perspective, overlap of secondary clinical features between the patient and the animal models provides additional critical evidence of a causal relationship between

candidate gene and disease. A key issue here remains the semantic representation of patient's phenotype and the use of a well-established phenotypic ontology during the examination processes. Explorations of secondary clinical features should be performed in future studies of genetic diseases.

Additional molecular screenings in larger populations of HPE patients are necessary to definitely assess the implication of our candidate genes in the disease. Therefore, we propose to include these novel genes into future genetic screenings of HPE patients.

In conclusion, this paper presents novel genes implicated in HPE and illustrates that HPE presents an oligogenic inheritance pattern requiring the joint effect of multiple genetic variants acting as hypomorphic mutations. The proposed inheritance pattern accounts for a wide clinical spectrum of HPE and explains the significant part of cases in which no molecular diagnosis could be established by conventional approaches. It also explains the incomplete penetrance and variable expressivity of inherited causal mutations observed in the reported cases of HPE (Mercier *et al.*, 2011). We propose that in cases of non-Mendelian diseases with variable phenotypes, the possibility of oligogenic inheritance needs to be evaluated. Exploration of such events will improve the diagnostic yield of complex developmental disorders and will contribute to better understanding of the mechanisms that coordinate normal and pathological embryonic development.

Acknowledgements

We would like to thank the families for their participation in the study, all clinicians who referred HPE cases, the eight CLAD (Centres Labellisés pour les Anomalies du Développement) within France that belong to FECLAD, French centers of prenatal diagnosis (CPDPN) and the SOFFOET for foetal cases, and the 'filière AnDDI-Rares'. We particularly thank all members of the Molecular Genetics Laboratory (CHU, Rennes) and of the Department of Genetics and Development (UMR6290 CNRS, Université Rennes 1) for their help and advice.

Funding

This work was supported by Fondation Maladie Rares (grant PMO1201204), Agence Nationale de la Recherche (grant ANR-12-BSV1-0007-01) and the Agence de la Biomedecine (AMP2016). This work was supported by La Fondation Maladie Rares and the Agence de la Biomedecine. The authors acknowledge the Centre de Ressources Biologiques (CRB)-Santé (http://www.crbsante-rennes.com) of Rennes for managing patient samples. This Work was supported by France Génomique National infrastructure, funded as part of "Investissement d'avenir" program managed by Agence Nationale pour la Recherche (contrat ANR-10-INBS-09) https://www.france-genomique.org/spip/spip.php?article158. This study makes use of data generated by the Genome of the Netherlands Project. Funding for the project was provided by the Netherlands Organization for Scientific Research under award number 184 021 007, dated July 9, 2009 and made available as a Rainbow Project of the Biobanking and Biomolecular Research Infrastructure Netherlands (BBMRI-NL). Samples where contributed by LifeLines (http://lifelines.nl/lifelines-research/general), The Leiden Longevity Study (http://www.healthy-ageing.nl; http://www.langleven.net), The Netherlands Twin Registry (NTR: http://www.tweelingenregister.org), The Rotterdam studies, (http://www.erasmus-epidemiology.nl/rotterdamstudy) and the Genetic Research in Isolated Populations program (http://www.epib.nl/research/geneticepi/research.html#gip). The sequencing was carried out in collaboration with the Beijing Institute for Genomics (BGI).

Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

Full collaborator details are available in the online Supplementary material.

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