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Abstract

Holoprosencephaly (HPE) is a complex genetic disorder of the developing forebrain characterized by high phenotypic and genetic heterogeneity. HPE was initially defined as an autosomal dominant disease, but recent research has shown that its mode of transmission is more complex. The past decade has witnessed rapid development of novel genetic technologies and significant progresses in clinical studies of HPE. In this review, we recapitulate genetic epidemiological studies of the largest European HPE cohort and summarize the novel genetic discoveries of HPE based on recently developed diagnostic methods. Our main purpose is to present different inheritance patterns that exist for HPE with a particular emphasis on oligogenic inheritance and its implications in genetic counseling.

INTRODUCTION

Holoprosencephaly is a severe developmental disorder classically defined as incomplete cleavage of the forebrain that originates from failed midline delineation during early development. There are several degrees of severity defined by the extent of the brain malformations. For the most severe cases, malformations are divided into alobar, semilobar or lobar forms. These brain abnormalities are associated with facial anomalies that are also of varying severity ranging from cyclopia to milder signs such as ocular hypotelorism. The full spectrum of HPE also includes microforms characterized by facial midline defects (e.g., single median incisor) without brain malformations typical of HPE (Cohen, 2006; Dubourg et al., 2007; Hahn, Barnes, Clegg, & Stashinko, 2010; Muenke & Beachy, 2000).

HPE occurs in most ethnic groups worldwide. Although implication of maternal diabetes in HPE has been reported (Barr et al., 1983), the well-established origin of HPE remains almost exclusively genetic and consists of chromosomal abnormalities and nucleotide-based variants (Dubourg et al., 2007, 2016; Solomon et al., 2012). So far, 17 genes have been implicated in HPE, all of which encode

proteins belonging to brain development pathways. *Sonic Hedgehog* (*SHH*) was the first discovered HPE gene (Roessler et al., 1996) and its alterations remain the most common cause of non-chromosomal HPE (Dubourg et al., 2016). *SHH* has been extensively studied and its functions during early brain development are now well described. A morphogenetic gradient of SHH is established from the ventral midline of the diencephalon to induce appropriate cleavage of both forebrain and eyefield. Remarkably, all HPE genes described so far are involved in the regulation of SHH activity (Sun et al., 2014; Xavier et al., 2016).

Initially described as an autosomal dominant trait with incomplete penetrance and variable expressivity, the mode of inheritance of HPE has been progressively redefined. The apparent autosomal dominant transmission with incomplete penetrance observed in a few HPE families may well be due to the cumulative effects of rare variants in two genes or more. Undeniably, the prevalence of oligogenicity has increased for several developmental pathologies since Next Generation Sequencing (NGS) technologies became accessible (Bamshad et al., 2011). Despite technical advances, defining the causative gene for HPE remains a difficult task, and even when one underlying variant is known, prenatal prediction remains uncertain.

Here, we describe clinical features and inheritance aspects of this disease with examples from our experience.

Clinical and genetics features of the cohort

The European HPE cohort

In 1996, a European HPE network was established in Rennes, France. Patients are recruited by clinicians from the different French centers of reference for rare developmental diseases as well as from several European clinical centers in the UK, Belgium, Italy, Spain and Portugal. Half of collected samples were fetuses, which enriched our cohort for severe HPE phenotypes (Mercier et al., 2011). Over the years, we have collected over 2,700 blood DNA samples or frozen fetal tissues, including patients and relatives, and gathered clinical data and DNA for 1,420 HPE probands. Our cohort contains both apparently sporadic and familial cases, excluding those associated with other malformation syndromes or with known chromosomal abnormalities that could be revealed by standard cytogenetic analysis (e.g., trisomies 18 and 13).

HPE presents a wide continuous spectrum of clinical malformations ranging from severe to milder forms. Our cohort is representative of the full clinical spectrum of HPE phenotypes (Mercier et al.,

2011). Within this cohort, the most severe brain malformations are categorized into alobar, semilobar or lobar form. Middle interhemispheric fissure (syntelencephaly) - incomplete separation of the posterior frontal and parietal regions - also belongs to the HPE spectrum (Figure 1). These brain abnormalities are significantly correlated with a variety of distinct facial anomalies ranging from cyclopia, the most severe form, to milder signs such as ocular hypotelorism. Severe facial phenotypes, such as cyclopia, ethmocephaly (proboscis) and cebocephaly are more highly associated with alobar HPE. Similarly, premaxillary agenesis, cleft lip or palate and milder ocular abnormalities (coloboma, retinal dysplasia) are mostly associated with semilobar HPE while the mild midface malformations, such as pyriform sinus stenosis and choanal stenosis are mostly found in lobar HPE. Patients who present the mildest facial abnormalities, such as hypotelorism, cleft lip or single median incisor, generally do not present easily detectable brain malformations (Mercier et al., 2011). These HPE microforms can nonetheless be associated with microcephaly and intellectual disability and their molecular diagnosis is therefore important for proper patient care (Bruel et al., 2017; Solomon et al., 2010). Notably, most of these microforms have been diagnosed only because they were relatives of patients with severe HPE. Some families manifest a wide range of phenotypes, from typical alobar HPE with perinatal lethality to microforms such as microcephaly, hypotelorism or both (Mercier et al., 2011). These different observations and clinical correlations made on a European cohort can be extended to all HPE cohorts, as they are very similar to those of North American patients (Lacbawan et al., 2009; Solomon et al., 2010).

As the frequency of microform HPE is underestimated, we are currently expanding our diagnostic approach to mildly affected relatives of a classical HPE patient. When a typical HPE patient is diagnosed in a family, we routinely perform a careful examination of all family members including neuroimaging techniques (MRI) and determination of clinical features that are not traditionally considered as a part of the HPE spectrum. Our goal is to expand our cohort to a larger number of HPE microforms to ensure that we cover the entire HPE spectrum.

The evolution of genetic strategies for HPE diagnosis

Sanger sequencing and detection of microdeletions in the major HPE genes

From the discovery of the first genes responsible for HPE until recently, genetic analysis of HPE patients has mainly relied on a Sanger sequencing approach (Mercier et al., 2011). During that period (1997 to 2010), screening our cohort for nucleotide-based variants in the four HPE genes – *SHH*, *Zinc Finger Protein 2* (*ZIC2), Six Homeobox 3 (SIX3)* and the Homeobox protein *TGIF1* – provided a global variant detection rate of 20 % (8.2 % for *SHH*, 7.4 % for *ZIC2*, 3.9 % for *SIX3* and 1.1 % for *TGIF1* (Mercier et al., 2011). All variants in these genes were detected heterozygously, and were shown to be loss-of-function variants (Roessler, El-Jaick, et al., 2009; Roessler, Lacbawan, et al., 2009). These

genes have constituted the four major genes of HPE. Meanwhile, novel genes have been implicated in sporadic cases of HPE (Table II and Roessler, El-Jaick, et al., 2009). The implication of each gene represents less than 1 % of HPE patients and they are therefore referred to as minor genes.

In 2003, variants in *GLI2*, one crucial effector of SHH signaling pathway (Ruiz i Altaba, Palma, & Dahmane, 2002), had been described in HPE patients (Roessler et al., 2003), and screening of our cohort (Figure 1) revealed *GLI2* variants in 3.2 % of the 302 patients tested (Mercier et al., 2011), thus placing *GLI2* as a major gene.

Identification of variants combined with detailed clinical assessment of HPE patients has allowed establishing some genotype-phenotype correlations (Mercier et al., 2011; Solomon et al., 2010). The most severe types of HPE (alobar and semilobar) tend to be associated with *ZIC2* and *SIX3* alterations while *SHH* tends to be more frequently associated with microforms. Remarkably, in *SHH* and *SIX3* cases, the facial dysmorphism is associated with brain anomalies while the probands of the *ZIC2* group tend to have a combination of severe HPE with few of the facial features. *GLI2* variants were preferentially found in patients presenting HPE microforms together with secondary specific features such as pituitary anomalies (Bear et al., 2014). These genotype-phenotype correlations have contributed to facilitate molecular analysis and genetic counseling for HPE.

Initially, Sanger sequencing has successfully allowed establishing molecular diagnoses for about 20 % of HPE patients present in our cohort (Mercier et al., 2011). In order to explain, at least part of the remaining 80 % of unsolved cases, we have searched for microdeletions in the major HPE genes (*SHH*, *ZIC2, SIX3* and *TGIF1)* first by quantitative multiplex PCR of short fluorescent fragments (QMPSF) and then by multiplex ligation-dependent probe amplification (MLPA). Deletions in HPE genes were thereby shown to be a common cause of HPE in up to 8.5 % of fetuses and in 5% in our whole cohort (Bendavid et al., 2006).

In 2006, the combination of these different approaches allowed us to diagnose molecularly 25 % of our patients, very similar to the success rate of our American colleagues (Roessler, El-Jaick, et al., 2009; Solomon et al., 2010, 2012). As 75 % of cases remained unsolved, we and others in the field have considered alternative genetic causes of HPE.

Chromosomal abnormalities and copy number variants in HPE

Since 2006, a pangenomic technique named comparative genomic hybridization (CGH) array has been used to screen the entire genome for copy number variations (CNVs). The first study we carried out during 2006-2009 revealed an impressively high rate of chromosomal rearrangements in HPE patients (22%), of which 14 % occurred *de novo* and 8 % were inherited (Bendavid et al., 2009, 2010). Furthermore, the observation of these CNVs can also lead to the detection of parental-balanced

translocations and can subsequently improve prenatal diagnosis in such families.

In addition, these CNVs involved novel potential HPE loci. Despite relatively low recurrence rates of CNVs, overlapping 6qter region deletions among 4 unrelated patients allowed identification of a ligand of the NOTCH signaling pathway, *Delta-like 1 (DLL1*) as candidate gene (Dupé et al., 2011). Subsequent detection of a nucleotide-based variant in a distinct patient provided further evidence for *DLL1* as HPE gene, and, together with expression and functional studies in vertebrates (Ratié et al., 2013; Ware, Hamdi-Rozé, & Dupé, 2014), allowed defining Notch as a novel signaling pathway involved in HPE.

In addition, microrearrangements found in unique cases, pointed to candidate genes such as *SIX6* and *OTX2*, which are both implicated in early brain development (Jean, Bernier, & Gruss, 1999; Jin, Harpal, Ang, & Rossant, 2001). Another study using a similar approach has also highlighted *OTX2* as a candidate gene (Rosenfeld et al., 2010).

Since this work, CGH-array has been and still is part of our systematic molecular screening of HPE patients. The novel detected CNVs are listed in Table II. The proportion of CNVs observed in this second period (2010-2017) is reduced as compared to that of our previous study (Bendavid et al., 2009). The rate of disease-relevant CNVs is now shown to be 10 %, half of which being *de novo*. Several of these rearrangements are recurrently observed in cases of intellectual disability, such as 2.6 Mb-microdeletion of 22q11.21 (proximal deletion) corresponding to DiGeorge syndrome (Burnside, 2015), 16p11.2 microduplication that confers susceptibility to autism (Fernandez et al., 2010), 16p13.11 encompassing the *NDE1* gene involved in brain neurogenesis and rhombencephalosynapsis (Bakircioglu et al., 2011; Démurger et al., 2013) and 15q11.2 microdeletion emerging as one of the most common cytogenetic abnormalities in intellectual disability and autism spectrum disorder (Butler, 2017). These deletions and duplications are thus at the origin of other neurodevelopmental disorders but are not sufficient to fully explain HPE. Nevertheless, CNV detection has increased the diagnostic yield from 25 % to 35 % in our cohort.

Next-generation sequencing (NGS) methods and their use for HPE diagnosis

The discovery of *SHH* in 1996 was followed by that of other genes - *ZIC2, SIX3, TGIF1* and *GLI2*. Since then, subsequent studies of the pathways implicating these major genes have contributed to the identification of additional HPE genes (Table I). Variants in genes involved in the SHH signaling pathway - *PTCH1, DISP1, CDON, GAS1, BOC* and *SUFU* - were described in some HPE patients (Bae et al., 2011; Dubourg et al., 2016; M. Hong et al., 2017; Ming et al., 2002; Pineda-Alvarez et al., 2012; Roessler, Ma, et al., 2009). A few variants have been described in *NODAL, TDGF1, FOXH1,* which encode proteins belonging to the Nodal/TGF-beta pathway (de la Cruz et al., 2002; Roessler et al., 2008; Roessler, Pei, et al., 2009). The Fibroblast Growth Factor pathway has also been implicated in

HPE through variants in *FGF8* and its receptor *FGFR1* (Arauz et al., 2010; Simonis et al., 2013). More recently, variants in *STIL,* a gene implicated in the formation of the primary cilia, were also described in HPE families (Kakar et al., 2015; Mouden et al., 2015). These are minor genes as they are reported in less than 1 % of HPE cases. Importantly, all these genes have in common the ability to affect SHH activity (Sun et al., 2014; Xavier et al., 2016).

Taking advantage of next-generation sequencing (NGS) in 2010, we established the first gene-panel sequencing method that targeted all known HPE genes (Dubourg et al., 2016). At the time we are writing this manuscript, more than 300 HPE patients have been tested. This study revealed that *SHH, ZIC2*, *SIX3 and GLI2* retain their position of major genes and *TGIF1* is relegated to the minor gene group. Furthermore, the identification of numerous variants in *FGF8* and *FGFR1* strengthens the involvement of FGF signaling in HPE (Figure 1B). Recent functional analysis in Zebrafish has confirmed the contribution of *FGF8* variants in HPE (Hong et al., 2016; Hong, Hu, Roessler, Hu, & Muenke, 2018) in accordance with the known function of FGF signaling during the specification of the dorso-ventral axis of the forebrain (Storm et al., 2006).

From the results we obtained over the years from our 1,420 probands, the 10 first-ranked genes involved are *SHH* (5.4%), *ZIC2* (5.2%), *GLI2* (3.2%), *SIX3* (3.0%), *FGF8* (2.5%), *FGFR1* (2.0%), *DISP1* (1.2%), *DLL1* (1.2%) and *TGIF1* (0.9%) (Figure 2; Table I). Some rare deleterious variants have been found in *SUFU*, a regulator of the Sonic-hedgehog-signaling pathway (Dubourg et al., 2016). By contrast, we did not detect any pathogenic variants in the following minor genes: *PTCH1*, *NODAL, GAS1, TDGF1, CDON,* and *FOXH1*. To date, only a small number of variants have been reported for these genes (Bae et al., 2011; de la Cruz et al., 2002; Ming et al., 2002; Roessler et al., 2008; Roessler, Pei, et al., 2009). Therefore, more data need to be collected to unambiguously assign these genes to HPE etiology.

As NGS technology evolved and became more accessible, whole exome sequencing (WES) is now routinely used to investigate novel HPE patients (see paragraph below). We expect this new approach will allow us not only to increase the diagnostic yield but also to identify novel HPE genes. Along the same lines, we are now establishing whole genome sequencing (WGS) approach in order to identify other alterations located in the noncoding part of the genome (~98 %) that has remained largely unexplored until now in the context of HPE.

An important observation has emerged from our experience as clinical reference center for HPE. When comparing the percentage of positive diagnoses between 2010 and 2017 it appears surprisingly stable (about 35 %), despite the fact that sequencing technology has improved and that

the number of HPE genes has increased. A closer examination of our data indicates that the fraction of patients with a CNV or with a variant in one of the major genes (*SHH, ZIC2* and *SIX3*) has been reduced by half as compared to that of 2010. Paradoxically, this reduction of cases is due to the increase of knowledge in diagnosis of HPE. Indeed, more and more clinical genetic centers are now able to perform their own molecular diagnoses for HPE. When a deleterious variant is found in one of the major HPE genes or when there is a CNV, the patient is not systematically referred to the reference center (i.e., Rennes, France) anymore. In contrast, when a molecular diagnosis could not be established, the patient data are sent to us for further investigation. As a consequence, the proportion of cases with an alteration in the major HPE genes has decreased in our cohort, which explains why our success rate of molecular diagnosis has remained stable over the years.

Modes of inheritance in HPE

In 1996, we initiated an epidemiologic study on 258 HPE cases and concluded that for non-syndromic and non-chromosomal HPE, the most compatible mode of transmission was autosomal dominant with incomplete penetrance and a variable expressivity (Odent, Le Marec, Munnich, Le Merrer, & Bonaïti-Pellié, 1998).

In HPE, the analysis of the first four major genes revealed that in most of the cases (70 % for *SHH* and *SIX3*) the variants were inherited from a parent who was asymptomatic or only mildly affected (microform HPE). By classic textbook definitions, autosomal dominant inheritance is defined as the transmission of disease from an affected parent to an affected offspring. In this model, half of this parent's offspring is expected to be affected. Our observations show that this model (and ratios of affected offspring) does not appear to apply to HPE. Furthermore, although as many as 17 genes have been linked to HPE, variants in these genes collectively explain only 25 % of all HPE cases. It suggests that HPE is a complex disease with an increasing number of causative genes for which inheritance can vary depending of the affected gene as well as other related factors. This observation stresses the need for clarifying potential modes of its inheritance. In this section, we present different cases from our cohort and discuss their corresponding inheritance pattern.

De novo versus inherited variants in HPE

Systematic sequencing of the major HPE genes has shown a high proportion of *de novo* variants in *ZIC2* (70 %), *SHH* (30 %) and *SIX3* (30 %) (Figure 3 and Mercier et al., 2011). Thus, *de novo* variants are implicated in numerous sporadic HPE cases. In accordance with the essential role of these genes in early brain development, these *de novo* variants are loss-of-function and tend to be more deleterious than inherited ones (Roessler, El-Jaick, et al., 2009).

In our cohort, 50 % of *FGFR1* variants appeared *de novo*. Adding to complexity, *FGFR1* is prone to mosaïcism as we could show in one family in which a patient's father presents an attenuated HPE phenotype (Dubourg et al., 2016). Interestingly, cases of mosaïcism involving *FGFR1* were described in other diseases like Hartsfield syndrome and encephalocraniocutaneous lipomatosis (Bennett et al., 2016; Dhamija et al., 2014). It should be noted that mosaic variants could be overlooked depending on the type of tissue tested and on the detection method (Braunholz et al., 2015), which could artificially increase the proportion of *de novo* variants.

Rare examples of autosomal recessive inheritance in HPE patients

In 2007, first case of recessive inheritance was described, involving two compound heterozygous variants in *TGIF1* (El-Jaick et al., 2007). Then, homozygous variant in *FGF8* was identified in one consanguineous HPE family (McCabe et al., 2011). More recently, another case of recessive inheritance involving *FGF8* was described (Hong et al., 2018). This *FGF8* variant was functionally validated and shown to be hypomorph, which is consistent with the indispensable role of FGF8 during early development (Sun, Meyers, Lewandoski, & Martin, 1999). A severe loss of function is probably not compatible with embryonic development.

Still, consanguineous families should predispose descendants to autosomal recessive gene combinations. Homozygosity mapping performed on 8 consanguineous HPE families from our cohort did not initially reveal any homozygous variants. Only by complementing mapping with WES we were able to detect in one of these families a homozygous hypomorphic variant for the gene *STIL* (Mouden et al., 2015). A homozygous nonsense variant of *STIL* was independently reported by others (Kakar et al., 2015). STIL is localized to centrioles where it participates in SHH signaling through its function in primary cilia biology and is known to be involved in microcephaly (David et al., 2014). Notably, in these two families, HPE is transmitted as a recessive trait associated with severe microcephaly. As no additional variant of *STIL* was described on more than 100 HPE patients tested (Karkera et al., 2002; Mouden et al., 2015), *STIL* gene therefore belongs to the minor HPE genes.

We also described a female HPE patient displaying two different mutated *DISP1* alleles both inherited from her two healthy parents (Mouden et al., 2016). These missense variants in the exon 10 were predicted to be deleterious. DISP1 is a protein that mediates the secretion of SHH, that is required for long-range cell to cell signaling (Tian, Jeong, Harfe, Tabin, & McMahon, 2005). This patient presents a localized fusion of forebrain hemispheres (mild form of HPE). We believe that hypomorphic effect of *DISP1* missense variant impacts SHH secretion to such an extent that global SHH signaling is decreased to pathological level.

All cases of autosomal recessive inheritance reported so far involve unaffected parents and concern minor HPE genes (Figure 3). Despite systematic sequencing of the major genes (*SHH, ZIC2* and *SIX3*)

in our 1420 probands and others (Roessler, Vélez, Zhou, & Muenke, 2012), no recessive case has been reported for these genes. It suggests that homozygous variants in major genes are not compatible with embryonic development, which is fully consistent with their crucial roles during early developmental stages (Geng & Oliver, 2009; Schachter & Krauss, 2008) and may explain why recessive inheritance is rare in HPE.

Oligogenic inheritance

As presented above, variants in HPE genes are frequently inherited from a parent without a typical HPE phenotype (Mercier et al., 2011). Within a given family we observed a large phenotypic heterogeneity between the different variant carriers, illustrating the incomplete expressivity of these variants (Kruszka, Hart, Hadley, Muenke, & Habal, 2015; Mercier et al., 2011; Solomon et al., 2010; Stokes et al., 2018). These observations support the hypothesis that in these families variant in one HPE-related gene is necessary but not sufficient for the disease to occur, which implies more variants are required for complete phenotypic spectrum. This oligogenic mode of inheritance has already been proposed by our colleagues, who referred to it as "automosal dominant with modifier effects" (S. Hong et al., 2016; M. Hong et al., 2017). In this oligogenic model, penetrance and expressivity of existing heterozygous variant is modulated by variants in other genes associated with HPE. Such a synergistic effect between distinct deleterious genetic events is now well-documented in several other hereditary developmental diseases (e.g, Alport, Bardet-Biedl and Kallman Syndromes) (Maione et al., 2018; Mencarelli et al., 2015; M'hamdi et al., 2014). In a similar manner, HPE could result from cumulated effects of distinct variants (Mercier, 2013). The use of animal models has reinforced this possibility. Numerous examples of double heterozygous mutant mice displaying HPE-like phenotypes provided evidence for oligogenism by implicating genes controlling either the same or distinct signaling pathways (Krauss, 2007).

For years, when a patient presented a deleterious variant in HPE gene, the analysis was interrupted because a likely genetic cause for the disease had been felt to be found. We believe it explains why rare HPE cases compatible with oligogenism have been reported so far (Ming & Muenke, 2002).

High-throughput sequencing and genomic technologies provided a unique opportunity to address this oligogenic inheritance in HPE. Thanks to WES, we have recently started to address the presence of additional events in HPE patients with a known variant in a HPE gene. Some of our most recent unpublished results on the subject are presented below.

Examples of digenic inheritance in HPE

Digenic inheritance is the simplest form of inheritance for genetically complex diseases. Systematic sequencing of major HPE genes, have described isolated cases with variants in two genes (e.g., *SHH/ZIC2*; *SHH/TGIF*) (Ming & Muenke, 2002). We also reported several cases of chromosomal rearrangement (CNV) associated with variant in HPE-related gene (Mercier et al., 2011).

In one family, presented in figure 2A, deleterious variants in both *SHH* and *DISP1* co-segregated with the disease, while relatives carrying variant only in either *SHH* or *DISP1* presented a mild or no disease phenotype (Mouden et al., 2016). This first digenic case in a family with several HPE patients prompted us to consider and further investigate digenic inheritance in families with HPE. This hypothesis was significantly reinforced by our experience with HPE genes routinely analyzed by targeted NGS. This study revealed that out of 257 HPE probands, 16 % of the variants used for diagnosis were found in association with a second variant (e.g., *FGF8/FGFR1*, *FGF8/DLL1*, *DLL1/SHH*, *DISP1/SUFU*) (Dubourg et al, 2016). More recently, two more cases of digenism were reported (*ZIC2/BOC* and *TGIF/BOC*) in HPE patients (Hong et al., 2017). Considering the systematic use of NGS on HPE patients and the increasing number of HPE genes, we expect that digenic cases will continue to accumulate further.

Cases of oligogenic inheritance in HPE

The increasing number of digenic cases provides novel insights into genetic etiology of HPE such as oligogenic inheritance. Oligogenic pattern has never been described in HPE, but is often suggested in other complex diseases such as ciliopathies (Reiter & Leroux, 2017), retinitis pigmentosa (Ali, Rahman, Cao, & Yuan, 2017), autism spectrum disorder (ASD) (Yin & Schaaf, 2017), amyotrophic lateral sclerosis (ALS) (Nguyen, Van Broeckhoven, & van der Zee, 2018), or porphyria (Lenglet et al., 2018). These disorders share with HPE high genetic and phenotypic heterogeneity as well as incomplete penetrance. We therefore considered oligogenic inheritance as a likely cause of HPE that may account for at least a substantial part of enigmatic cases.

With constantly evolving knowledge of disease genes and emergence of new analysis methods, it has become important to us to reanalyze systematically previous unsolved cases. In that aim, we reevaluated unsolved HPE families by taking into account the possibility of oligogenic inheritance. The study included families with no identified causal variant as well as families with variants in *SHH, ZIC2, SIX3 or TGIF1* inherited from clinically unaffected/mildly affected parent. Parts of this unpublished work are presented and discussed hereafter.

We combined trio-based whole exome sequencing with deep clinical phenotyping of the patients. Variant analysis was further improved by a gene prioritization approach based on clinical ontologies and co-expression networks of known disease-related signaling pathways.

In one family, presented in Figure 2B, we identified a variant in *SHH,* which was inherited from the mother presenting with hypotelorism. Considering oligogenic inheritance, we addressed whether paternal variants could contribute to HPE phenotype in combination with the *SHH* variant inherited from the mother. Indeed, our analysis revealed paternally inherited variants in two candidate HPE genes - *FAT1* and *NDST1*. Both variants were rare (minor allele frequency below 1 %) and were predicted deleterious by the majority of bioinformatics algorithms (CADD score > 20). FAT1 is a protocadherin and its knockdown in mouse causes severe midline defects including HPE (Ciani, Patel, Allen, & ffrench-Constant, 2003) and NDST1 is an N-deacetylase sulfotransferase and the corresponding mice mutants exhibit reduced SHH signaling and HPE-like phenotype (Grobe, 2005). Additionally, clinical phenotyping and analysis of cross-species similarities provided further evidence of causality for these genes by revealing a strong overlap of clinical features between the patient and the *FAT1* (proboscis) and *NDST1* (eye defects) mutant mice. Finally, the segregation analysis showed that the combination of variants in *SHH/FAT1/NDST1* was exclusively found in the two affected individuals of this family (Figure 2B). This example nicely illustrates oligogenic inheritance in HPE where the disease results from accumulation of multiple variants in genes associated to HPE phenotypes and/or implicated in SHH signaling (Figure 3). We therefore pursue a systematic reevaluation of all unsolved HPE cases. We believe that numerous other genes will be characterized in patients with oligogenism transmission. An exciting future challenge will be to test experimentally the combined effect of these different variants on early brain development.

Our clinical approach

Over the last two decades, we have followed for prenatal diagnosis 26 HPE families affected by a first case of severe HPE carrying a variant in one of the major HPE gene. In 18 instances, we were able to reassure the parents after establishing the absence of the gene alteration in the fetus. Fetal MRI scan was normal later in pregnancy, and no child had medical problems after birth. A genetic alteration (in *SHH*, *SIX3* or *TGIF1*) was found in the 8 other cases: 5 children were born, either without brain malformation and asymptomatic, or presenting a less severe form than the proband as predicted by the fetal brain surveillance. Three pregnancies were interrupted after MRI scans showed HPE features.

Nowadays, even when a causative gene for HPE has been found in a patient, the molecular diagnosis is probably not fully established. In order to properly address the molecular diagnosis, it will be necessary to compare the detailed phenotypes of the different family members with the segregation of relevant rare variants. For practitioners involved in counseling, an important consideration is how to communicate results of genetic analysis when potentially deleterious variants are identified but

not yet functionally validated. Ideally, determining the contribution of each variant to the phenotype would be a condition for reliable genetic counseling in HPE families with oligogenic transmission.

Conclusion

In this review, we aimed to present the different patterns of inheritance of HPE in the light of our experience (Figure 3). In some cases, the disease is due to *de novo* variants; in rare cases the disease exhibits classical Mendelian inheritance with autosomal recessive transmission. In most cases, it emerges that the penetrance and the phenotypic variability have digenic or oligogenic origin. This complex genetic architecture will be better understood by analysis of hundreds of genes with NGS techniques on unsolved HPE cases. Our future challenge will be to differentiate rare variants that have significant impact on the observed phenotype from those with no effect.

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Figure legends

Figure 1. Clinical and molecular details of the European HPE cohort

(A) Brain anomalies distribution in European HPE cohort (1,420 probands).

(B) Mutational spectrum of HPE.

In blue, number of variants found in 2010. 164/642 (25.4 %) patients were found to harbor variants in *SHH*, *ZIC2*, *SIX3* or *TGIF1*. *GLI2* variants were identified in 3/208 patients (Mercier et al., 2011).

In red, number of variants found in 2017. Molecular screening of major HPE genes in 1,420 patients revealed 207 variants in *SHH*, *ZIC2*, *SIX3* and *TGIF1*. Complementary screenings on a series of 302 patients revealed 32 variants in *DLL1, FGF8, FGFR1, DISP1* and *GLI2*.

(C) Contribution of CGH-array to molecular diagnosis. In 2010, CNVs were detected in 22 % of the 260 patients analyzed by CGH-array, including 36 occurring *de novo*. In 2017, the screening of our entire cohort (1,420 patients) reported CNVs in 142 patients (10%), including 71 occurring *de novo*.

Figure 2. Examples of complex inheritance in HPE.

(A) Family presenting a digenic mode of transmission associating variants in *SHH* and *DISP1* (Mouden *et al.,* 2016). Minor signs refer to microcephaly.

(B) Family presenting an oligogenic pattern with combined inherited variants in *SHH*, *FAT1* and *NDST1*. Minor signs refer to hypotelorism (father) and epicanthus (mother).

Individuals marked with asterisk were analyzed by whole exome sequencing. NA: Not available for *DISP1* sequencing.

Figure 3. Different inheritance patterns for HPE are presented together with illustrative cases from our unpublished data.

TABLE I. List of HPE genes and corresponding percentages of variants found in our HPE cohort.

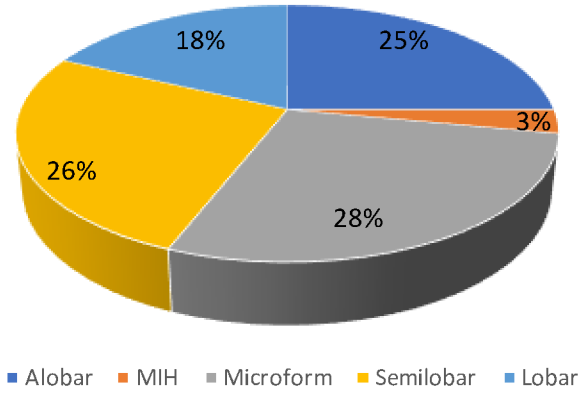
Chromosome	Gene	NM	%
7	*SHH*	000193.2	5.4
13	*ZIC2*	007129.3	5.2
2	*GLI2*	005270.4	3.2
2	*SIX3*	005413.3	3.0
10	*FGF8*	033163.3	2.5
8	*FGFR1*	023110.2	2.0
1	*DISP1*	032890.3	1.2
6	*DLL1*	005618.3	1.2
18	*TGIF1*	170695.2	0.9
10	*SUFU*	016169.3	0.4
1	*STIL*	001048166.1	1 case/375
9	*GAS1*	002048.2	0
3	*TDGF1*	003212.3	0
11	*CDON*	016952.4	0
8	*FOXH1*	003923.2	0
10	*NODAL*	018055.4	0
3	*BOC*	001301861.1	Not tested

TABLE II: Summary of chromosomal abnormalities in HPE cases

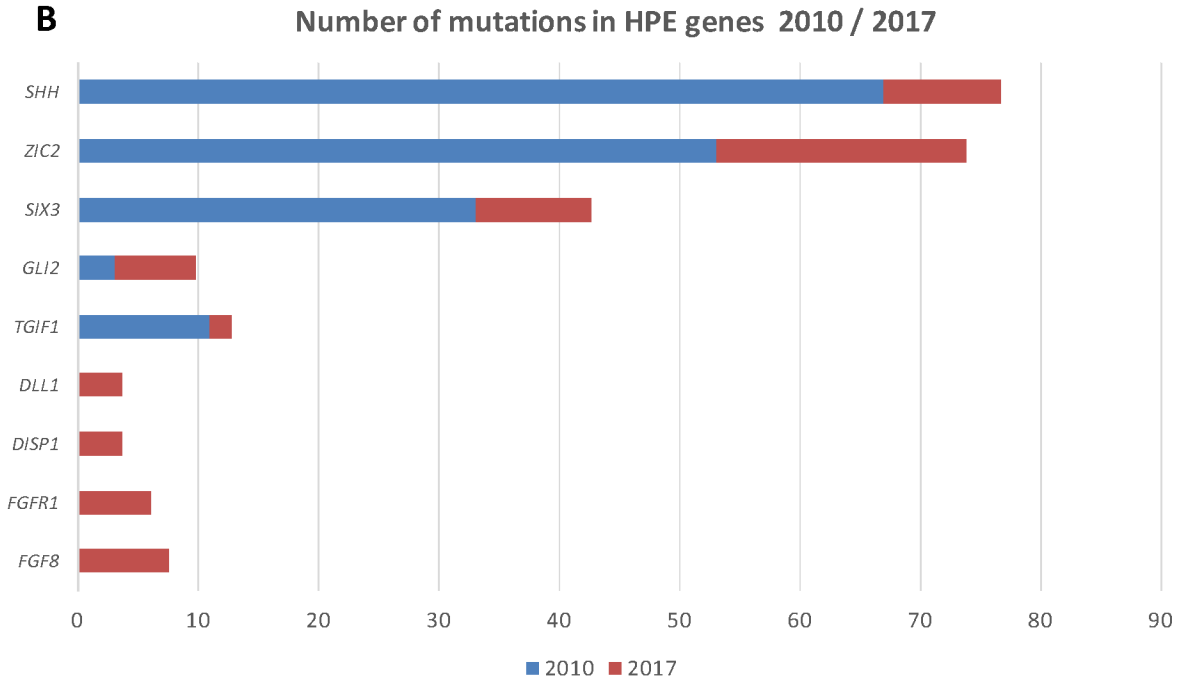
Patient	Cytoband	Type of CNV	Start-End (GRCh37)	CNV size	Inheritance
1	1q43q44 3p25.2p22.1	del dup	242094954_ 249212668 0_ 39848444	7 Mb 40 Mb	Inherited from parental balanced t(1;3)
2	2p15	del	61668439_ 61777447	109 kb	*De novo*
3	2p11.2 16p13.11*	dup dup	85824180_ 86469217 15492317_ 16276115	645 kb 784 kb	*De novo* Inherited from father
4	3p22;20q11.2	balanced translocation	-	-	Inherited from mother
5	3q25.32	del	157105931_ 157154842	50 kb	Inherited from mother
6	4q12	del	55193357_ 58196685	3,00 Mb	ND
7	5q35.3	del	177296851_ 178323802	1 Mb	Inherited from mother
8	5q35.3	dup	178038828-179766520	1,73 Mb	Inherited
9	7p22.1	del	5399371_ 6871084	1,4 Mb	*De novo*
10	7p22.1	dup	5057686_ 5166175	108 kb	Inherited from father
11	8q23.3q24.11	del	117641330-118051191	410 kb	ND
12	Whole chromosome 8	dup in mosaic	0_ 146294098	146 Mb	*De novo*
13	10p15.3p14	del	136361-9946915	9,8 Mb	*De novo*
14	11q13.4	dup	74931870-75109882	178 kb	Inherited from mother
15	12q21.32	del	86984993_ 87656628	672 kb	ND
16	14q23.1	del	60950490_ 61006021	55 kb	Inherited from mother
17	14q23.1	dup	Unavailable data	900kb	Inherited from mother
18	15q11.2*	del	22765628-23208901	443 kb	Inherited from mother
19	16p13.11* 16p11.2*	del dup	15492317_ 16267306 29652999_ 30197341	1,3 Mb 544 kb	ND Inherited from mother
20	18q22.1	dup	64152648_ 64324336	172 kb	Inherited from mother
21	19q13.42q13.43	dup	56228025_ 57744093	1,5 Mb	*De novo*
22	22q11.21*	del	20719112-21464119	745 kb	ND
23	22q11.21*	del	21081060_ 21505558	424 kb	*De novo*
24	22q11.21*	del	18894835_ 21464119	2,6 Mb	*De novo*
25	22q11.21*	del	18894835_ 21464119	2,6 Mb	*De novo*
26	22q11.21*	del	18651614_ 21801661	3,2 Mb	ND
27	Xq25q28	del	123176394-152515593	29,34 Mb	*De novo*

del = deletion; dup = duplication; ND = not determined; *CNV (Copy number variation) usually observed in intellectual deficiency

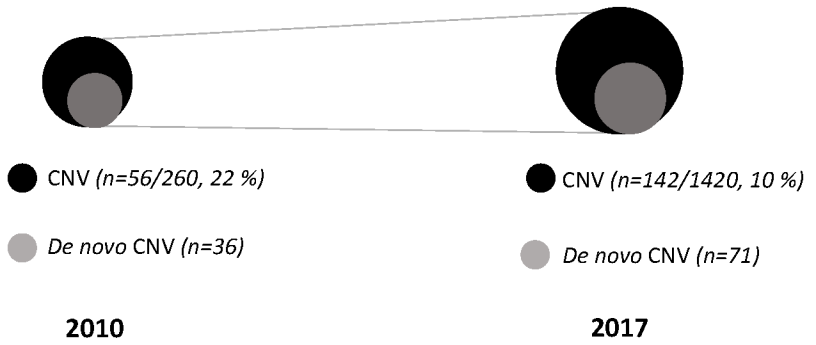
A **Distribution of brain anomalies in European HPE cohort**

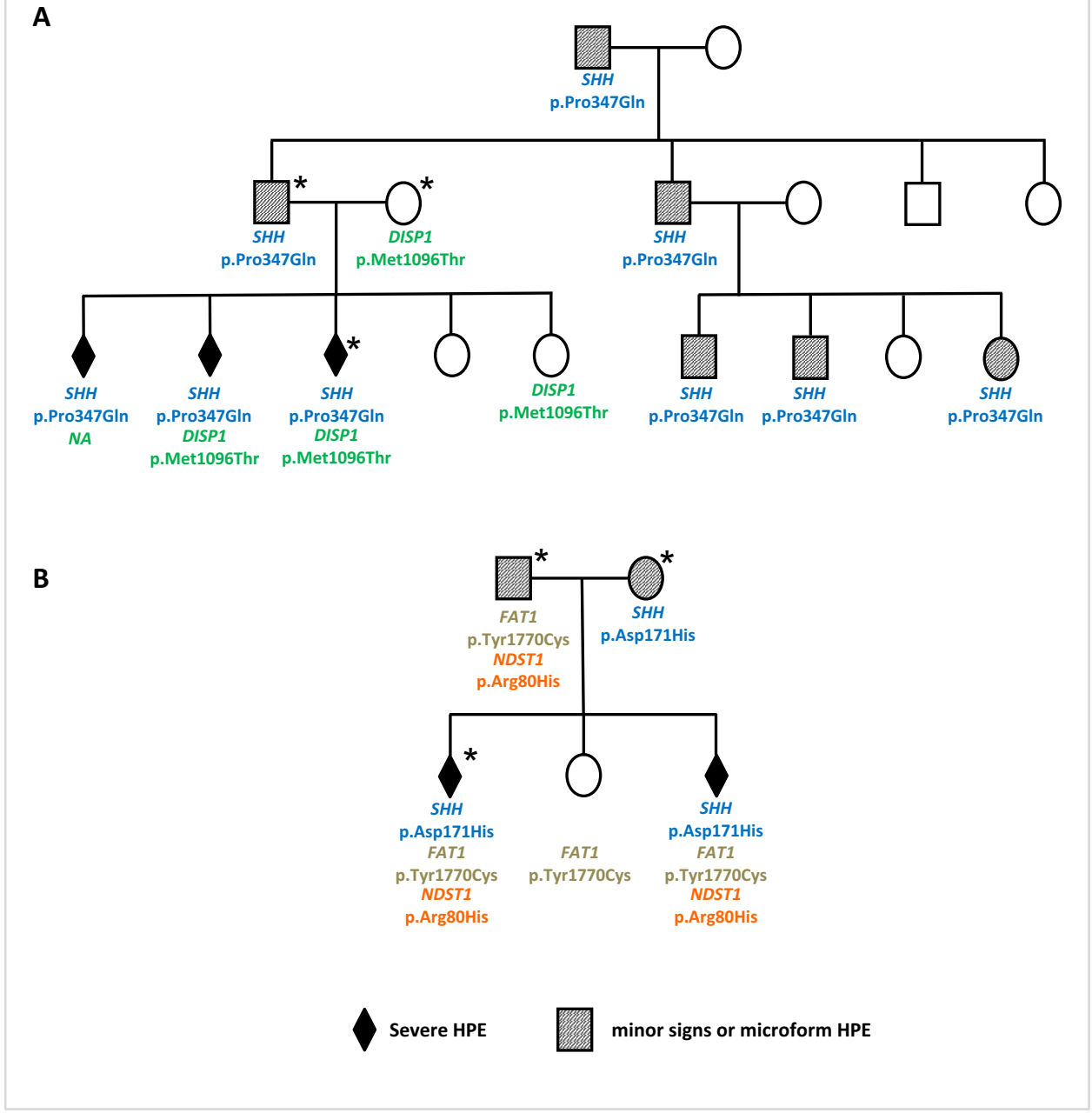


B



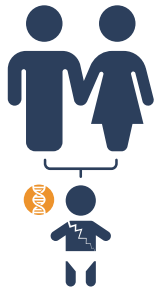
C




A
SHH
p.Pro347Gln
SHH
p.Pro347Gln
DISP1
p.Met1096Thr
SHH
p.Pro347Gln
SHH
p.Pro347Gln
NA
SHH
p.Pro347Gln
DISP1
p.Met1096Thr
SHH
p.Pro347Gln
DISP1
p.Met1096Thr
DISP1
p.Met1096Thr
SHH
p.Pro347Gln
SHH
p.Pro347Gln
SHH
p.Pro347Gln
B
FAT1
p.Tyr1770Cys
NDST1
p.Arg80His
SHH
p.Asp171His
SHH
p.Asp171His
FAT1
p.Tyr1770Cys
NDST1
p.Arg80His
FAT1
p.Tyr1770Cys
SHH
p.Asp171His
FAT1
p.Tyr1770Cys
NDST1
p.Arg80His
Severe HPE
minor signs or microform HPE

Autosomal dominant *(de novo)*

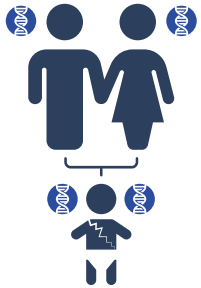
~35 %



ZIC2 (70%)
FGFR1 (50%)
SHH (30%)
SIX3 (30%)

Autosomal recessive

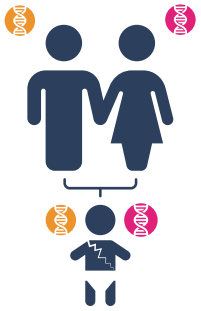
<1 %



STIL/STIL
DISP1/DISP1
TGIF1/TGIF1
FGF8/FGF8

Digenism

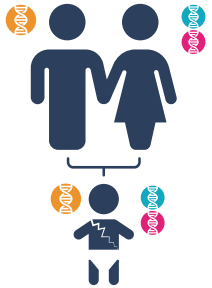
? %



SHH/DISP1
SIX3/PTCH1
SHH/DLL1
FGF8/FGFR1
…

Oligogenism

? %



SHH/FAT1/NDST1
…