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Holoprosencephaly: an update on cytogenetic abnormalities

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ABSTRACT

Holoprosencephaly (HPE), the most common developmental defect of the forebrain and midface, is caused by a failure of midline cleavage early in gestation. Isolated HPE, which is highly genetically heterogeneous, can be due to major chromosomal abnormalities. Initially, karyotype approach led to the identification of several recurrent chromosomal anomalies predicting different HPE loci. Subsequently, several genes were isolated from these critical HPE regions, but point mutations and deletions in these genes were found only in 25% of the genetic cases. In order to identify other HPE genes, a more accurate investigation of the genome in HPE patients was necessary. To date, high-resolution cytogenetic techniques such as subtelomeric multiplex ligation-dependent probe amplification (MLPA) and microarray-based comparative genomic hybridization (array CGH) have enhanced chromosomal aberration analysis.

In this article, we have updated the cytogenetic anomalies associated with HPE in a map listing all the subtelomeric and interstitial deletions that have been characterized either by karyotype, MLPA or array CGH. The accumulation of recurrent genomic imbalances will lead to the further delineation of minimal critical HPE loci, which is the first step to the identification of new HPE genes.

KEY WORDS: holoprosencephaly, HPE, array CGH, MLPA, candidate loci molecular diagnosis.

INTRODUCTION

The etiology of holoprosencephaly (HPE) is heterogeneous and complex, as this developmental disorder can be due to environmental factors, chromosomal aberrations, or genetic anomalies. In this chapter, we focus on the chromosomal aberrations that have been seen in HPE patients.

Chromosome analysis constitutes an excellent approach to identifying the cause of genetic syndromes, particularly for central nervous system (CNS) malformations [Shaffer and others 2007]. The first cytogenetic anomalies reported in HPE were trisomy 13, trisomy 18, and triploidies [Norman 1995; Roessler and Muenke 1998]. Subsequently, advances in cytogenetic techniques such as chromosome banding allowed for higher resolution and for the description of chromosomal anomalies in a collection of patients with HPE. Estimates of the prevalence of karyotype anomalies in live births range from 24% to 45% [Bullen and others 2001; Croen and others 1996; Olsen and others 1997]. Numerous isolated HPE case reports show that most of the chromosomes have been implicated, emphasizing the genetic heterogeneity of HPE [Norman 1995; Roessler and Muenke 1998].

Despite this heterogeneity, extensive studies on patients with brain malformations and autosomal deletions demonstrate that four main regions are associated with HPE [Brewer and others 1998; Tyshchenko and others 2008]. Molecular studies of these four recurrent chromosomal regions have resulted in the definition of a minimal critical region and ultimately in the identification of HPE-specific genes. The implication of these regions in HPE was confirmed by the discovery of specific point mutations or deletions in the following genes: Sonic

Hedgehog, *SHH* (7q36) (HPE3) [Belloni and others 1996; Dubourg and others 2004; Roessler and others 1996], *ZIC2* (13q32) (HPE5) [Brown and others 2001; Brown and others 1998], *SIX3* (2p21) (HPE2) [Gripp and others 2000; Pasquier and others 2000; Wallis and others 1999], and *TGIF* (18p11.3) (HPE4) [Aguilella and others 2003; Gripp and others 2000].

These genes have since constituted the four major genes implicated in the susceptibility to HPE, but have only been found to explain 25% of the genetic cases, including deletions and microdeletions [Dubourg and others 2007]. This suggests the involvement of other genes, a hypothesis later confirmed by the identification of numerous additional genes: *GLI2* (HPE9) [Rahimov and others 2006] [Roessler and others 2003], *PATCHED-1* (HPE7) [Ming and others 2002; Roessler and others 2003], *DISP1* [Roessler and others 2009a; Roessler and others 2009b], *FOXH1* [Roessler and others 2008], *NODAL* [Roessler and others 2009c], and *TDGF1* [de la Cruz and others 2002] (Figure 1). However, mutations or deletions in these genes occur infrequently and are generally associated with HPE microforms. In fact, dysfunction of only one of these genes appears to be not sufficient to cause severe features of typical HPE. Consequently, these genes are thought to have a minor overall effect (present review). Considering this multigenic aspect of the disease, investigation of HPE loci and identification of new HPE genes need to continue.

Originally, the altered chromosomal regions described previously overlapped with one or several G bands and were generally too large to allow for the identification of single candidate genes. Use of molecular screening techniques such as subtelomeric multiplex ligation-dependent probe amplification (MLPA) or microarray-based comparative genomic hybridization (array CGH) has enhanced chromosomal aberration analysis. In the last five years, these techniques have been

used to delineate critical genomic regions, and their high resolution capabilities have accelerated the identification of novel chromosomal abnormalities. In this article, we summarize the new deleted loci identified by these newer cytogenetic methods and refine the HPE locus map.

THE STATE OF THE KARYOTYPE APPROACH

Previous analysis of recurrent chromosomal deletions detected by karyotype led to the identification of 12 HPE candidate loci [Norman 1995; Roessler and Muenke 1998]. Subsequently, several genes were isolated from these critical HPE regions, leading to a new nomenclature. The HPE loci previously identified as HPE2, 3, 4, 5, 7 and 9 (OMIM) are now named by their corresponding genes: *SIX3*, *SHH*, *TGIF*, *ZIC2*, *PTCH1* and *GLI2*, respectively. In addition, there are three recurrent HPE regions that are also considered most likely to be candidate HPE loci, although no specific genes have been identified. These regions have been listed by OMIM as HPE1 (21q22), HPE6 (2q37) and HPE8 (14q13).

In this paper, we have designed a new chromosomal map for HPE to account for these recent changes (Figure 1). This map schematizes the validated HPE gene positions and the cytogenetically abnormal regions for which no specific candidate genes have been identified to date.

This map also represents isolated karyotype abnormalities in 1pter, 3q, and 5pter that may also contain potential candidate genes for HPE [Campeau and others 2008; Schroeder and others 1986; Simovich and others 2008]. It is necessary to exhaustively record all the chromosomal rearrangements that could be validated by further studies; nonetheless, only deletions are reported here in the interest of clarity.

IDENTIFICATION OF SUBTELOMERIC GENOMIC IMBALANCES BY MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA)

Bendavid *et al.* demonstrated that testing for microdeletions is a useful approach in the evaluation of HPE patients as this method increased the diagnosis efficiency from 18% to 25% of this cohort. However, since these studies were based on FISH, real time quantitative PCR (qPCR) and quantitative multiplex PCR of short fluorescent fragments, these pilot evaluations were restricted to known HPE genes [Bendavid and others 2006a; Bendavid and others 2006b].

Of note, two of the microdeleted genes were subtelomeric (*TGIF* and *SHH*), constituting some of the previously described HPE loci [Roessler and Muenke 1998]. Such subtelomeric rearrangements were successfully identified in several studies on mental retardation using a PCR based method named multiplex ligation-dependent probe amplification (MLPA) [Koolen and others 2006; Northrop and others 2005]. These studies led to the conclusion that submicroscopic rearrangements in the subtelomeric regions played an important role in the etiology of congenital defects [Rauch and others 2006] [Ledbetter and Martin 2007].

Therefore, using the same method, subtelomeric screening of HPE patient samples was performed to identify new rearrangements in HPE patients. First, Bendavid *et al.* tested 10 HPE patients with known deletions: eight *SHH* deletions (7q36) and two *TGIF* deletions (18p) [Bendavid and others 2007]. They observed that the previously described microdeletions were not restricted to the gene itself, but instead encompassed several Mb from the gene to the telomere. Further qPCR analyses showed that the size of the 7q36 deletions was about 7 Mb in the four

patients tested. Moreover, five of the 7q36 deletions were associated with gains on another telomere: two on 7p, two on 8p and one on 1p. This also resulted in the identification of a parental balanced translocation that could be used secondarily for prenatal diagnosis.

Subsequently, a panel of 181 HPE patients without any known chromosomal anomaly was tested and subtelomeric aberrations were detected and confirmed in 8/181 HPE cases (4.4%, see green bars in Figure 1). One patient had a deletion on 21q, or the HPE1 locus, where no gene is yet firmly identified. The lanosterol synthase gene (*LSS*) was considered to be the best candidate, but its direct involvement in HPE was never demonstrated [Roessler and others 1999]. Also of note, two unrelated patients showed deletions in 1pter, a region that has been further implicated by karyotype analysis [Campeau and others 2008]. For the five other patients, a gain and/or loss of chromosomal material was detected in novel subtelomeric regions, not previously reported as candidate HPE loci by karyotype analysis (Figure 1): a 1q gain, a 5q deletion associated with a 17q gain, an 18q deletion associated with an Xq gain, a 15 subcentromeric gain, and a 20p gain with a 22q deletion [Bendavid and others 2007].

The subtelomeric MLPA technique has proven to be a good approach to detect new chromosomal aberrations in HPE patients and confirms that submicroscopic rearrangements were implicated in this disorder. Thus, genome-wide screening for submicroscopic anomalies were needed to test for the presence of rearrangements in the whole genome.

IDENTIFICATION OF SUBMICROSCOPIC REARRANGEMENTS BY MICROARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION (ARRAY CGH)

Novel genomic technologies such as microarray-based comparative genomic hybridization (array CGH) allow for the mapping of genomic copy number alterations at submicroscopic levels. The resolution of these arrays has progressively increased from 1 Mb, using BAC arrays, to approximately 8 kb, when using high density oligonucleotide arrays. Notably, studies on large cohorts of patients with mental retardation and associated facial dysmorphisms were performed using array CGH and have delineated the major HPE loci in accordance with the previous karyotype results [Shaffer and others 2007]. Therefore, this technology has been applied to a cohort of HPE patients and to case reports.

In this way, a group of 111 HPE patients (64 fetuses and 47 live-born children) with normal karyotype was analyzed using high performance Agilent oligonucleotide arrays [Bendavid and others 2009]. Among these patients, all fetuses had characteristic HPE phenotypes (CNS findings consistent with HPE) whereas of the 47 live-born cases, 32 (68%) had microforms of HPE and 15 (32%) had a severe form. Chromosomal imbalances not described in the CNV databases were detected in 28 patients (17 fetuses, 11 children) (25%), out of which 19 had *de novo* anomalies (17% of the whole cohort). In total, of these 19 patients with *de novo* gains or losses, 13 presented isolated deletions, only four showed isolated duplications, and two demonstrated associated gains and losses. Considering that the clinical impact of duplications is more difficult to establish and that gene duplications are less

deleterious than deletions in HPE cases [Bendavid and others 2009], we will only discuss here the potential implications of the genome losses.

Deletions were found in the 21qter and 14q loci, which are previously annotated in the literature [Kamnasaran and others 2005; Roessler and others 1999], reinforcing their potential involvement in HPE. Several new candidate loci not suspected to be involved in the disease at that time were also identified: some were interstitial deletions in 1p, 6q, 10p, 16p, 18q, 20p, 21q and Xp; others were subtelomeric deletions in 19pter and 6qter (Figure 1). The size of these imbalances ranged from 50 kb to 17 Mb without an obvious correlation with the severity of the phenotype [Bendavid and others 2009].

Although most of these deletions were not redundant, two of them – one in 6qter and one in 10p – were observed in more than one HPE case. Notably, the two overlapping deletions in 6qter mapped a new region, but their large size makes the identification of new candidate genes difficult. In fact, the smallest deletion contains at least 35 genes or putative open reading frames. More significantly, the small (0.1 Mb) deletions observed in 10p12.1 were detected in four HPE patients and contained *PATCHED3*. This gene belongs to the SHH receptor family and thus represents an excellent candidate. Nevertheless, no point mutation has been found in a cohort of 100 HPE patients (data not published, CD). Also notably, five of the non-redundant deletions are small in size. For instance, the 19pter locus is about 0.4 Mb in size and contains only six genes; unfortunately, none of them is known to be implicated in brain development.

Other deletions included a hemizygous deletion of about 10.4 Mb in size that was detected on another 6q band (6q22-6q23) in an isolated case of middle interhemispheric variant (MIHV) HPE [Abe and others 2009]. In this large area

comprising many genes, the authors have considered *EYA4* as a new HPE candidate gene based on its expression during forebrain development and on its protein interaction with SIX3 [Abe and others 2009]. Nevertheless, only the identification of point mutations or microdeletions in HPE patients will definitively validate the participation of *EYA4* in HPE.

THE IMPACT OF ARRAY CGH IN HOLOPROSENCEPHALY RESEARCH

The array CGH study conducted on the HPE cohort clearly demonstrates that microcytogenetic abnormalities are a frequent cause of HPE with 17% of de novo anomalies. In addition, this high frequency, regardless of the size, location, and redundancy of the rearrangements, demonstrates that array CGH is an important tool to detect submicroscopic molecular defects in HPE patients.

Nevertheless, the interpretation of the non-recurrent rearrangements should be cautious and their clinical relevance should take into account several parameters. First, the presence of potential copy number polymorphisms (CNP), which account for over 20% of the human genome, should be ruled out by comparison with the Database of Genomic Variants (<http://projects.tcag.ca/variation/>), which compiles most known CNPs. Second, it is crucial to test for the presence of chromosomal anomalies in the parents, as it can be postulated that only anomalies that are not associated with a mutation and that arise *de novo* in the proband are relevant. Alternatively, considering that HPE is a multigenic disease with variable expressivity, inherited genetic anomalies can contribute to an HPE genetic background and thus these inherited deletions should also be recorded (Figure 1, light blue). Finally, due to positional effects, genes localized outside but close to a rearrangement should not be

discarded but instead be considered as candidate genes. Indeed, it was recently shown that not only hemizygous genes but also normal-copy neighboring genes can show decreased levels of expression [Merla and others 2006]. Therefore, genes flanking a genomic rearrangement should be considered as possible contributors to the phenotype. In addition, the interpretation of these observations is not obvious, as most of the chromosomal aberrations are large. One strategy would be to use software dedicated to the prioritization of candidate genes [Aerts and others 2006] [Tranchevent and others 2008], followed by functional analysis using animal models and research of mutations in these genes.

Beyond these technical pitfalls, array CGH has been shown to be a powerful tool that significantly helped in the identification of genes involved in various pathologic conditions. The first instance was the identification of the gene responsible for CHARGE syndrome, following the localization of a deletion at an 8q breakpoint in an apparently balanced translocation [Vissers and others 2004]. A more recent example is a collaborative work leading to the identification of a new gene (*MEF2C*) involved in a specific form of mental retardation. In this case, array CGH led to the characterization of a minimal common deleted region in 5q14 in five patients, encompassing the gene *MEF2C*. Afterward, the identification of a *MEF2C* nonsense mutation in another patient supported its pathologic role [Le Meur and others 2009].

In HPE, the same strategy may lead to a better delineation of minimal critical regions in large recurrent chromosomal deletions that presently do not contain obvious candidate genes or where the major gene implicated to date is controversial. As an example, the 18p deletions are overrepresented among HPE chromosomal aberrations [Overhauser and others 1995], whereas a low rate of *TGIF* mutations (less than 1%) is observed in HPE patients [Dubourg and others 2004]. This would

suggest that this subtelomeric region should be investigated in detail in order to search for other HPE genes. In fact, in the context of a multigenic disease, the implication of several genes in the onset of HPE has to be strongly kept in mind.

Ultimately, using array CGH routinely will enrich the present map (Figure 1) and the catalog of redundant deleted regions will lead to the further delineation of HPE candidate loci, which is the first step to the identification of new HPE genes.

SUMMARY

Here, we have updated the cytogenetic anomalies associated with HPE in a map listing all the subtelomeric and interstitial deletions that have been characterized either by karyotype, MLPA or array CGH. Considering the high rate of such anomalies observed in HPE, this technique must be integrated in the molecular diagnosis algorithm. The observation of cytogenetic anomalies in patients can lead to the detection of parental balanced translocations, and can subsequently enhance prenatal diagnosis in such families.

This type of compilation must be systematically updated in the next years and will constitute a reference database either for the scientific community or for clinicians who will consult it for diagnostic assistance.

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Figure 1: Diagrammatic representation of the localization of HPE deletions and HPE genes on G banded chromosomes.

Kaki bars on the left side represent deletions detected by routine karyotype. Green bars on the right side represent rearrangements identified by subtelomeric multiplex ligation-dependent probe amplification (MLPA), with dotted lines where the deletion is not bordered. [Bendavid and others 2007]. Blue bars on the right side represent submicroscopic deletions identified by microarray-based comparative genomic hybridization (array CGH): dark blue denotes *de novo* deletions, while light blue shows inherited deletions [Abe and others 2009; Bendavid and others 2009]. To demonstrate an exhaustive repertory of the genetics of HPE we have positioned the major (red) and minor (orange) HPE genes: CHR 1 [Campeau and others 2008; Roessler and others 2009b], CHR 2 [Lehman and others 2001; Pasquier and others 2000; Rahimov and others 2006], CHR 3 [de la Cruz and others 2002; Lawson-Yuen and others 2006; Simovich and others 2008], CHR 5 [Schroeder and others 1986], CHR 7 [Belloni and others 1996], CHR 8 [Roessler and others 2008], CHR 9 [Roessler and others 2003], CHR 10 [Roessler and others 2009c], CHR 13 [Brown and others 1998], CHR 14 - HPE8 [Kamnasaran and others 2005], CHR 18 [Gripp and others 2000], and CHR 21 - HPE1 [Roessler and others 1999].