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# **Our current recommendations for the molecular evaluation of newly diagnosed holoprosencephaly patients**

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### ABSTRACT

Holoprosencephaly (HPE) is the most common structural malformation of the developing forebrain in humans and is typically characterized by different degrees of hemispheric separation that are often accompanied by similarly variable degrees of craniofacial and midline anomalies. HPE is a classic example of a complex genetic trait with “pseudo”-autosomal dominant transmission showing incomplete penetrance and variable expressivity. Clinical suspicion of HPE is typically based upon compatible craniofacial findings, the presence of developmental delay or seizures, or specific endocrinological abnormalities, which is then followed up by confirmation with brain imaging. Once a clinical diagnosis is made, a thorough genetic evaluation is necessary. This usually includes analysis of chromosomes by high-resolution karyotyping, clinical assessment to rule-out well recognized syndromes that may cause HPE (e.g. Pallister-Hall syndrome, Smith-Lemli-Opitz syndrome and others), and molecular studies of the most common HPE associated genes (e.g. *SHH*, *ZIC2*, *SIX3*, and *TGIF*). In this review, we will provide our current step-by-step recommendations that are medically indicated for the genetic evaluation of patients with newly diagnosed HPE. Moreover, we will provide a brief review of the several available methods used in the molecular diagnostics of HPE and describe the advantages and limitations of both currently available and future tests as they relate to high throughput screening, cost, and the results that they may provide.

**KEY WORDS:** holoprosencephaly, HPE, disease genes, multi-factorial inheritance, molecular diagnostics

## **INTRODUCTION**

Holoprosencephaly (HPE) is most common disorder of the developing forebrain in humans, and it occurs with a frequency of 1:250 conceptuses [Matsunaga and Shiota, 1977] and 1:10-16,000 live births [Roach et al., 1975]. The HPE phenotypic spectrum results from failure of the forebrain to cleave into two hemispheres. Different degrees of hemispheric separation, ranging from the classically described alobar form, to semilobar, lobar and middle-interhemispheric variant (MIHV) describe the anatomically distinguishable forms of HPE. The mildest end of the spectrum includes subtle midline brain anomalies. These phenotypes are often accompanied by a broad spectrum of craniofacial differences, ranging from the most severe form with cyclopia (one eye) or synophthalmia (two fused eyes) with a proboscis (nose-like appendage), to less severe forms with hypotelorism, mid-face hypoplasia or a single maxillary central incisor (SCI) [Cohen, 2006; Dubourg et al., 2007; Muenke and Beachy, 2000; reviewed in Solomon et al., this issue]. The occurrence and manifestations of HPE are influenced by both genetic causes and environmental risk factors. In cases where a specific gene is known to be causative, it is inherited as a typical complex trait with incomplete penetrance and variable expressivity. The basis of these phenotypic differences is largely unknown but likely reflects measured and unmeasured genetic and environmental components [Solomon et al., 2009].

## **CYTOGENETIC ALTERATIONS AND MUTATIONS OF DEVELOPMENTAL GENES ARE THE MOST COMMON KNOWN CAUSES OF HPE**

It is estimated that the etiology of HPE is due to cytogenetic anomalies in 30-50% of individuals, is associated with well recognized syndromes (e.g. Smith-Lemli-Opitz syndrome (SLOS)) in ~25%, is due to either environmental causes and/or unknown genetic alterations in ~10-15%, and is caused by mutations in established HPE gene(s) ~5-10% [Bullen et al., 2001; Ong et al., 2007; Dubourg et al., 2007; Roessler et al., 2009a]. Additional risk factors that may act alone or in concert with genetic alterations include the use of retinoids, statins, or alcohol during pregnancy, alterations in the biosynthesis of cholesterol, and pre-existing or gestational diabetes [Cohen and Shiota, 2002].

Mutations in at least 12 genes have been detected in patients with HPE, however, there is significant variability in the observed mutation rate of each gene (see below). Therefore, our current recommendations will reflect those genes with the highest degree of clinical utility for study, and we will propose which genes are best left to research applications. The most common HPE genes were identified as mutational targets within loci defined by chromosomal rearrangements [Dubourg et al., 2004; Muenke and Beachy, 2000]. Among the best characterized HPE genes are *SHH* [Roessler et al., 1996], *ZIC2* [Brown et al., 1998], *SIX3* [Wallis et al., 1999], *TGIF* [Gripp et al., 2000], *GLI2* [Roessler et al., 2003], *PATCHED-1* [Ming et al., 2002], and *DISP1* [Roessler et al., 2009c], and others. Most commercial and research laboratories only screen the first four genes (the named HPE loci 2-5) for mutations on a routine basis. Micro-deletions and micro-duplications have been suggested to play important roles given that some of these alterations occur in the vicinity of known HPE genes [Bendavid et al., 2009].

Currently, there are still a large proportion of individuals with non-syndromic and non-chromosomal HPE (~75% of patients) in which no specific genetic cause can be identified [Wallis and Muenke, 2000]. The general consensus regarding the etiology of HPE is that the molecular interactions and pathways are complex [Monuki, 2007], consistent with the theory that a large number of loci or genetic factors are yet to be identified and more fully understood. The primary goal of this review is to describe our current recommendations for molecular genetics testing of patients with newly diagnosed HPE, the types of strategies for evaluation that are currently used, what tests are likely to be of use in the future, and the advantages and limitations of these technologies.

### **OUR CURRENT EVALUATION STRATEGY**

As described in Table I, methods such as single strand conformational polymorphism (SSCP) and denaturing High Performance Liquid Chromatography (DHPLC) have been used in the past as effective screening methods [Brown et al., 1998; Gripp et al., 2000; Roessler et al., 1996; Wallis et al., 1999]. However, disadvantages of SSCP and DHPLC include their comparatively low sensitivity and the exposure of the laboratory environment to radioactivity (SSCP) and the requirement of volatile chemicals to perform analysis (DHPLC) [O'Donovan et al., 1998; Orita et al., 1989]. New technologies, such as next generation platforms, offer the potential for more comprehensive analysis of a larger number of genes with even greater sensitivity and specificity (see below).

The clinical diagnosis of HPE is confirmed by a combination of physical examination, family history, and brain imaging (MRI, CT, or ultrasound, etc.). Once

HPE is confirmed, we recommend that parents be invited to participate in the subsequent genetics work-up in order to allow for a more complete family-based evaluation in the setting of a positive cytogenetic or molecular finding. Parental participation is also beneficial in terms of the broader goal of fostering research in the field of HPE research even if the given test results in their particular case are unrevealing. Our experiences over the past decade have proven that the value of cooperation amongst multiple international testing centers goes beyond simply being able to share methodologies and testing strategies, but that the sharing of patient data and test samples enhances our likelihood of identifying additional HPE genes in the future.

As shown in Figure 1, we propose a general strategy for the genetic evaluation of a newly diagnosed patient with HPE. Holoprosencephaly is usually diagnosed clinically based upon specific phenotypic features (described above) [Cohen, 2006; Dubourg et al., 2007; Orioli and Castilla, 2007], which typically must then be confirmed with brain imaging in order to fully characterize the anomaly [Hahn and Plawner, 2004]. A comprehensive evaluation of a patient with HPE should typically begin with cytogenetic studies, including a high-resolution karyotype with a minimum of 550 band resolution, given that ~40-50% of patients will have a chromosomal anomaly [Cohen, 2006; Orioli and Castilla, 2007]. In selected patients, medically indicated studies should then be done to rule out syndromes that might cause HPE (e.g. 7-dehydro-cholesterol levels elevated in SLOS). Finally, in all non-syndromic patients found to have normal chromosomes, molecular analysis should be performed for the most common genes implicated in HPE: *SHH*, *ZIC2*, *SIX3* and *TGIF* [Dubourg et al., 2004; Wallis and Muenke, 2000].

It is at the initial evaluation of a proband that it is most appropriate for the primary care giver to assess the willingness of the parents to participate in research in HPE, both by enrolling their child in a research study and by providing samples of their own blood for parallel studies. Analysis of parental samples will further be important for the interpretation of the proband's test results and for future genetic counseling, whether or not a cytogenetic or molecular diagnosis is immediately established. The strongest predictors of the pathogenicity of new alterations relates to whether the changes are *de novo* gross cytogenetic, occult microgenetic, or mutations [reviewed in Roessler and Muenke in this issue]. In the broader research context, the accumulation of a diverse set of parent-child trios allows for future studies to address new genetic associations, modifier screens, and other methods aimed at better understanding how genetic interactions and genetic variations may influence the variable penetrance and expressivity of HPE traits. Hence, the participation of parents in the molecular evaluation of their children can have both direct and indirect benefits for HPE research.

For the above patients who have an abnormal karyotype, the cytogenetic findings should be correlated with the clinical phenotype and the underlying mechanism involved. For example, well recognized trisomies in chromosomes 13 and 18 or other rearrangements that may disrupt one of the major genes implicated in HPE, such as *SHH* or *ZIC2*, and thus contribute to the etiology of HPE [Dubourg et al., 2007]. Other chromosomal rearrangements can also occur [see Roessler and Muenke, this issue], however currently there is little proof of the pathogenicity for the majority of them. New technologies, controlled population genetics, and functional studies should allow us to further expand our knowledge. Again, parental studies are important to define whether

the anomaly is segregating through the family or if it is a *de novo* event. A more in-depth molecular analysis of the chromosomal breakpoints, using DNA sequencing or array-CGH, can be important given that, the vicinity of the breakpoints produces unstable DNA with deletions and duplications frequently occurring beyond the particular locus. This additional research will allow for better characterization of the genetic alterations and phenotypic correlations, which may be helpful for genetic counseling purposes.

For the patients with a normal karyotype, DNA sequencing analysis should be performed for the most commonly identified genes associated with HPE. In general, mutations in *SHH* are present in ~12% of affected patients [Roessler et al., 2009a], *ZIC2* in ~9% [Roessler et al., 2009b], and *SIX3* in ~5% [Lacbawan et al., 2009]. Given the high detection rate of likely pathologic mutations, we consider these genes to be essential for a first line medical assessment. Other genes have been described to be associated with HPE, such as *TGIF* (altered in ~1% of patients) [Gripp et al., 2000; Wallis and Muenke, 2000], *GLI2* (~1%) [Roessler et al., 2003], *PATCHED-1* [Ming et al., 2002], *DISP1* [Roessler et al., 2009c], *FOXH1*, *NODAL* [Roessler et al., 2009d], and others. However, at the present time, we recommend that these latter genes with low mutation frequency rates among HPE patients be tested only in select cases, or that they be referred to specialized testing centers with the requisite expertise.

One example of a specialized situation that calls for testing of *GLI2* is when abnormalities occur in the development of the pituitary gland, in the context of variable brain and craniofacial anomalies consistent with the broad spectrum of HPE [Roessler et al., 2003; Roessler et al., 2005]. Likewise, other genes have also been shown to be associated with characteristic brain and craniofacial abnormalities [Solomon et al., this

issue; Muenke Lab unpublished data]. In the special case where a specific phenotype is present, molecular analysis of the associated locus is considered medically indicated. However, we still strongly encourage enrollment in research studies given that additional genetic and/or environmental co-factors may also be playing a role, even when a mutation is present in a well-characterized gene.

From our current molecular diagnostic perspective, exonic mutational analysis via bi-directional DNA sequencing remains the gold standard. Both pay-for-service and free research options are available and give comparable results. Independently of where patients elect to do their molecular diagnostic test, we recommend that clinicians encourage patients to freely enroll in a HPE research study. For example, in the consent process for research into HPE and related brain disorders at the National Institutes of Health (NIH), we offer clear opt-out provisions for future research results that empower parents to set limits on the results that they wish to obtain now, and those generated by future studies. Likewise, we also recommend families to join support groups such as “Families for HoPE,” which will help them to overcome the difficulties of management of patients with the disorder.

When a novel mutation is identified in a proband, such as, a single nucleotide change, insertion, duplication, deletion or a frame-shift mutation, parental samples should subsequently be tested to assess whether the mutation is segregating in the family (familial HPE) [Solomon et al., 2009] or a *de novo* variant. In general, *de novo* mutations are more likely to be pathogenic based on functional studies [Domené et al., 2008]. However, a large proportion of patients have unique mutations that are family-specific that can make it very difficult to predict the likely consequences [Roessler et al., 2009a].

Therefore, continued cooperation with research centers can often result in the development of functional tests for novel sequence variants that can clarify the nature of such alterations.

In order to better identify which genetic variants are truly pathogenic, the identified variants must be correlated with their predicted or experimentally determined residual function. Computerized prediction algorithms may be used, however they may be inconclusive; therefore, highly specialized functional studies based on animal models, cellular models and conservation analyses among vertebrate species are typically required. Functional consequences of changes in *SIX3* [Domené et al., 2008], *SHH* [Roessler et al., 2009a], *ZIC2* [Roessler et al., 2009b], and *TGIF* [El-Jaick et al., 2007], have been well illustrated [reviewed in Roessler and Muenke, this issue].

Not all variants among the HPE genes are obvious loss-of-function. Although nucleotide changes occurring in very conserved regions of the genome are more likely to cause defects through loss-of-function, further analyses are frequently necessary to determine their precise effects [Kryukov et al., 2007]. Importantly, there is also increasing evidence that gene regulatory elements and non-coding portions of HPE genes can play an important role in disease causation and would be missed by most traditional diagnostic strategies [Jeong et al., 2008].

Local genetic counseling, facilitated by the expertise of tertiary care centers and patient groups, should be offered to families whether results of the genetic tests are negative or positive. This counseling should be guided by state-of-the-art evidence to help to interpret the results and their limitations. When there is inconclusive evidence about the effect of a given variant, it should be made clear to the family that the effect is

uncertain. As noted previously, this is another strong case for participation in research investigations in both mutation positive and mutation negative cases.

### **PAST, PRESENT AND FUTURE METHODS AND THEIR IMPLICATIONS**

In Table I, we present the advantages and disadvantages of several methods that have been used and that are being proposed for the molecular study of HPE. In the past, SSCP and DHPLC have been used to screen for mutations in patients. SSCP was initially the best way to pre-screen individuals for variants for a given DNA product, however it was not an ideal technique given that the radioactive materials required special handling and training and constituted a potential hazard for the laboratory environment, and that the sensitivity of the test was low [Orita et al., 1989]. DHLPC was presented as an alternative method, as it had improved sensitivity, provided higher throughput options than SSCP, and the preparation, run and analysis of the experiments were relatively short [O'Donovan et al., 1998]. The individual loading of samples was bypassed with a semi-automated plate analysis system.

Capillary electrophoresis DNA sequencing is the current gold standard for mutational screening of HPE genes, with its primary advantage being close to 100% sensitivity and specificity. However, data analysis is labor intensive and challenging given the presence of ambiguities that may occur in the chromatograms, as well as allelic drop-out and failure to detect deletions/duplications that are larger than the sequence being interrogated. Although DNA sequencing is more readily available than other technologies, and it can be used with equal success on both medically-indicated and

research-only genetic tests, there is still a strong need for newer methods given the extensive heterogeneity of causative HPE genes.

Multiplex ligation-dependent probe amplification (MLPA) (MRC-Holland, Amsterdam) is a relatively new molecular method to detect the occurrence of micro deletions/duplications in genes. There is a panel commercially available (SALSA MLPA kit P187 Holoprosencephaly – MRC-Holland, Amsterdam, Netherlands) with probes spanning the HPE genes [Bendavid et al., 2009]. Among the limitations of this method are that it is available in only a few laboratories, a follow-up test is necessary to validate presence of the loss/gain of dosage (e.g. qPCR), and it is unable to detect single nucleotide mutations or smaller deletions or duplications. There is sufficient evidence in the literature of an overwhelming number of single nucleotide mutations or small deletions/duplications causing truncated proteins. For example, in a recent study on patients with HPE and alterations in *SHH*, there were 125 different mutations in individuals with holoprosencephaly tabulated [Roessler et al., 2009a]. Hence, copy number variations and hypothetical promoter or enhancer variations are likely to be among the least common types of variations that are likely to be detected.

High-resolution DNA melting (HRM) strategies have recently been proposed to pre-screen samples for mutations [Reed et al., 2007]. In our experience, amplicons from many individuals can be simultaneously screened from genomic DNA in roughly two hours, followed by direct sequencing of a targeted subset of presumed variants. This method promises considerable savings in terms of money and time in the identification of variants. Some of its greatest advantages are that it has high sensitivity and, specificity, (over 95% for heterozygous variants) [Wittwer, 2009] and the high throughput nature it

allows up to 384 samples to be screened per run, in a Roche LightCycler 480 II instrument (Roche Applied Science, Indianapolis, IN and Idaho Technology Inc., Salt Lake City, UT). As with any new technology, there are also disadvantages. HRM loses efficacy with screening GC-rich amplicons due to the difficulty in denaturing them. However, denaturing solutions, such as Roche GC-RICH solution (Roche applied science, Indianapolis, IN), can be added to enhance the melting process to increase the specificity [Tindall et al., 2009]. Moreover, current protocols recommend that amplicons be limited in size, up to 400 base pairs [Wittwer, 2009].

Next-Generation (NextGen) sequencing strategies and array-CGH (aCGH) offer the promise of great amounts of information [Bejjani and Shaffer, 2006; Mardis, 2008] although it is not yet clear which of the many new methods will emerge as the most useful. With both of these new strategies, the interpretation of results should be made carefully, since miscalling of normal variants as mutations presents the risk of misinterpretation. Currently, there is not enough evidence from well-controlled studies to unambiguously differentiate disease-causing alterations from incidental copy number variants except for the ones involved in the known HPE genes [Bendavid et al., 2009]. Further research use should help to mitigate these obstacles. Since these techniques are so new and rapidly changing, most of the technologies have not been FDA approved for routine clinical use, nevertheless, they are currently used by commercial diagnostic laboratories, such as GeneDx (Gaithersburg, MD, USA) for the diagnostic studies of several diseases. Array-CGH may not be appropriate for use on a routine basis until there is a better understanding of the implications of copy number variants (CNVs) in the pathogenesis of HPE. As with all detection methods, presumptive positive results should

be followed up by family studies, since occurrence of novel events are more likely to be pathogenic.

New technologies, such as HRM, aCGH and NextGen Sequencing, will allow for the generation of large amounts of data with sensitivities and specificities over 90%, the ability to detect CNVs that we were not able to previously identify, and for the routine screening of more genes and regulatory elements to be both cheaper and faster. However, the generation of such overwhelming amounts of data by itself does not always translate into a better understanding of a disorder. Consequently, the application of these tests in a clinical context is presently limited [Bejjani and Shaffer, 2006].

We recommend the formation of a worldwide consortium, where research data, DNA samples and cell lines would be shared between the largest possible number of active investigators involved on HPE research, in order to accomplish an integration of knowledge which would contribute to a thorough understanding of the clinical and genetic aspects of this disease. While no such formal organization yet exists, the rationale for such an effort is clear. The extensive genetic heterogeneity of HPE and the unresolved issues underlying its characteristic variable expressivity compel researchers in this field to cooperate with one another and to enlist the cooperation of primary care providers, patient groups and families in this effort. Some of the obvious future challenges of this proposed group will be to collect cases for large-scale studies (e.g. to establish routine functional studies based on animal or cellular models, perform family-based association studies, and case-control association studies) to dissect the genomic variants that impact on HPE incidence and severity. Large datasets increase the statistical power of such studies and enhance the certitude of the interpretations. Such an approach,

in combination with the technologies mentioned above, should allow, in the future, the expansion of a more comprehensive genetic testing strategy of patients with the HPE phenotypic spectrum and their relatives.

Finally, all of these considerations contribute to difficulties in counseling families with HPE [see Odent, this issue]. The extreme heterogeneity and diverse manifestations of HPE presents considerable challenges to medical geneticists and counselors. No single algorithm is presently sufficient to explain all cases of HPE. However, we hope that by providing a guideline for the busy clinician we can inspire the genetics community to engage in fostering important research in this area. The sharing of cases and case materials should maximize the ability of clinicians to provide meaningful results to their patients for the present, as new technologies offer the future promise of an even greater understanding of this complex set of malformations.

## **SUMMARY**

In summary, our current recommendations of medically indicated genetic testing of families with HPE are: cytogenetic studies as the first layer of the algorithm (see review by Bendavid et al., this issue), since cytogenetic abnormalities make up the most common causes of HPE. Molecular testing of *SHH*, *SIX3* and *ZIC2* are the second layer of evaluation, since they explain at least 20% of non-syndromic and non-chromosomal HPE. Other genes identified in HPE should be tested as complementary studies in special cases, given their low frequency (~1% or less). These steps should take place in the context of a discussion about whether to pursue commercial lab testing and/or enrollment in a research study.

## **DISCLOSURES**

The authors have no competing financial interests to declare.

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Table I.

Use	Method	Advantages	Disadvantages
Past	SSCP	<ul style="list-style-type: none"> <li>• A popular, rapid, inexpensive screen for nucleotide variants</li> <li>• Detects presence of normal and variant alleles</li> <li>• Heterozygous vs. homozygous results obvious</li> </ul>	<ul style="list-style-type: none"> <li>• Lowest sensitivity and specificity</li> <li>• Small amplicons</li> <li>• Use of acrylamide gels and radioactivity</li> <li>• Requires confirmation with sequencing</li> </ul>
	dHLPC	<ul style="list-style-type: none"> <li>• Semi-automated</li> <li>• Higher throughput capacity and sensitivity than SSCP</li> <li>• Requires less time and labor than SSCP</li> <li>• Improved cost profile</li> </ul>	<ul style="list-style-type: none"> <li>• Specificity still marginal</li> <li>• Requires confirmation with sequencing</li> </ul>
Present	Automated capillary DNA sequencing	<ul style="list-style-type: none"> <li>• Gold Standard</li> <li>• ~100% sensitivity and specificity</li> <li>• Semi-automated</li> <li>• High throughput capacity</li> </ul>	<ul style="list-style-type: none"> <li>• Requires significant investigator edits</li> <li>• Ambiguities frequent, occult allelic drop</li> <li>• Typically fails to detect large deletions or duplications</li> </ul>
	HRM	<ul style="list-style-type: none"> <li>• Effective screening method</li> <li>• High throughput</li> <li>• Sensitivity over 90%</li> <li>• Specificity excellent and improves with increased throughput</li> <li>• Post-PCR manipulation is not required</li> <li>• Fast automatic run where the analysis can focus on sequencing the uncommon variants flagged by the software</li> </ul>	<ul style="list-style-type: none"> <li>• Screening of some GC-rich regions can be challenging</li> <li>• Optimal results with small amplicons ~300 bp</li> <li>• Requires follow-up sequencing of variants</li> </ul>
	MLPA	<ul style="list-style-type: none"> <li>• Fast and high throughput method</li> <li>• Detects sub-microscopic deletions/duplications missed by sequencing</li> </ul>	<ul style="list-style-type: none"> <li>• Typically requires validation studies</li> <li>• Few laboratories perform test</li> </ul>
	Array-CGH	<ul style="list-style-type: none"> <li>• Capable of detection of genome wide gains/losses of copy</li> <li>• Requires no hypothesis</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive</li> <li>• Validation with another method often needed</li> <li>• Only large scale changes</li> <li>• Needs several micrograms of DNA</li> </ul>
Future	Next-Generation Sequencing	<ul style="list-style-type: none"> <li>• Capable of genome-wide individualized data</li> <li>• High tiling path = few errors</li> <li>• Unambiguous results</li> <li>• Relatively fast</li> </ul>	<ul style="list-style-type: none"> <li>• In development on multiple platforms</li> <li>• Huge amounts of data (almost all of which is normal)</li> <li>• Significance of most variants will initially not be understood</li> </ul>

**Figure 1. Algorithm for the genetic study of new holoprosencephaly patients:** Bold lines refer to medically indicated tests; thin lines are optional tests depending on a specific clinical indication or the capabilities of the diagnostic laboratory; dotted lines refer to tests available in research labs that will contribute to a better understanding of HPE. For further details, see the following references: **a:** [Hahn et al.,this issue; [Hahn and Plawner, 2004] **b:** [Dubourg et al., 2007] **c:** [Roessler and Muenke, this issue] **d:** [Bendavid et al., this issue] **e:** Refers to High-Resolution DNA Melting (HRM) **f:** Multiplex Ligation-dependent Probe Amplification (MLPA) **g:** gene specific phenotype, **h:** [Bullen et al., 2001; Ong et al., 2007].

