Molecular prenatal diagnosis of a sporadic alobar holoprosencephalic fetus: genotype-phenotype correlations

Jean Gekas[1], Consolato Sergi[2], Deepak Kamnasaran[3]

[1] Human Genetics Research Unit, Centre de recherche de l'Hôpital Saint-François d'Assise, Québec, Canada
[2] Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Canada
[3] Department of Pediatrics, Laval University, Québec, Canada

Corresponding author:
Deepak Kamnasaran
Department of Pediatrics
Laval University
RC9800, 2705 boulevard Laurier
Québec, QC, Canada
G1V 4G2,
Phone: (418) 656-4141
Fax: (418) 654-2753
E-mail: deepak.kamnasaran@crchul.ulaval.ca

Summary

Objective: **holoprosencephaly is the most common forebrain malformation syndrome with a multifactorial etiology. Currently, mutations are identified in 5-10% of non syndromic, non-chromosomal cases in at least 12 genes. We report the molecular prenatal diagnosis of a fetus with alobar holoprosencephaly.**
Methods: **CTG band karyotyping and array CGH genome-wide cytogenetic screenings were done, in conjunction with DNA sequence analyses of the *SHH*, *ZIC2*, *SIX3* and *TGIF* genes in search of a molecular etiology and with comparison of findings to prior cases.**
Results: **standard CTG band karyotyping and array CGH genome-wide screening failed to identify plausible chromosome imbalances or structural anomalies. However, extensive sequencing of the genomic DNA from the fetus and both parents on all exon and exon-intron boundaries of the four most commonly mutated genes: *SHH*, *ZIC2*, *SIX3* and *TGIF*, identified codon 100 of the sonic hedgehog (*SHH*) gene having a hotspot for loss-of-function mutations in our case and others.**
Conclusion: **mutations in codon 100 of *SHH* were discovered in both sporadic and autosomal dominant inherited cases with evidence of variable expressivity and penetrance. Collectively, this study reinforces the complexity of genotype-phenotype correlations in the prenatal diagnosis of holoprosencephalic fetuses.**

Key words: genetic testing, pregnancy, craniofacial dysmorphology, birth defect, genetic counseling.

Introduction

Holoprosencephaly (HPE) is the most common malformation associated with multiple congenital anomalies (MCA) syndrome with a principal defect in the developing prosencephalon. In humans, the incidence is estimated at 1 in 250 conceptuses and 1 in 10-16,000 live newborn infants (1). The pathogenesis of HPE involves a failure of the prosencephalon to properly cleave into the two cerebral hemispheres resulting in forebrains with variable severity and generally defined by the extent of the midline longitudinal fissure, fusion of the thalami and the holosphere is horseshoe-shaped cavity containing a single ventricle that opens postero-dorsally. The spectrum includes the most severe alobar and semilobar types and the least severe lobar variant, in which the fissure can be detected cleaving the cerebrum into two cerebral hemispheres more caudally only (1,2). These forebrain malformations are often accompanied with a spectrum of facial findings, with cyclopia and cebocephaly being the most severe, and with lesser severe forms including midfacial hypoplasia, single maxillary central incisor and hypotelorism. In the majority of cases (>70%), the severity of concurrent forebrain and facial anomalies are positively correlated. Current prenatal diagnostic protocols on the clinical management of holoprosencephaly involve combined fetal imaging analyses [2D and 3D ultrasonography in first or second trimester (3-5) or MRI] with cytogenetic and molecular genetic testing on suspected cases. However, the current limitation of prenatal fetal imaging is a bias detection of the most severe forebrain and facial anomalies as early as the first trimester, along with poor sensitivity in detecting mild disease manifestations such as lobar holoprosencephaly (6). An effective improvement of the diagnosis of holoprosencephalic brain anomalies becomes evident by the third trimester only (6). Holoprosencephaly is also considered a complex trait with a multifactorial etiology often resulting in incomplete penetrance, variable expressivity and spurious genotype-phenotype correlations. Therefore, these overall complexities can often impinge on accurate prenatal diagnosis and genetic counseling. The inheritance of nonsyndromic HPE is typically autosomal dominant with high but incomplete penetrance (~82%) (6). However, the vast majority of affected cases are sporadic. To date, 12 HPE loci are defined in the human genome (1). Be-

tween 25-50% of syndromic cases have a cytogenetic anomaly (1). Moreover, mutations were identified in 5-10% of nonsyndromic, non-chromosomal HPE cases in at least 12 HPE genes (1). Overall, the most common mutations are documented in *SHH* in ~12% of affected subjects, and to a lesser extent ~9% in *ZIC2*, ~5% in *SIX3* and ~2% in *TGIF* (1). All causative genes have crucial developmental functions in the patterning of the developing prosencephalon and associated facial structures, although mutations of the SHH gene are particularly intriguing in light of the role of this gene in neuroectodermal induction. Improvements in the fetal diagnosis and genetic counseling of holoprosencephaly necessitate ongoing molecular prenatal diagnostic studies. To accomplish this, we report a new fetal case manifesting holoprosencephaly and with important mutational findings.

Case report

This study has institutional ethics approval. Our proband (Fig. 1A) was conceived by a 37-year-old healthy mother (2 Para, 1 Abortus) and is the second child of a non-consanguineous family. The first child is clinically normal. The family history is unremarkable. No drugs or infections were reported during the course of the pregnancy. At 15 gestational weeks, alpha fetoprotein levels were detected three times higher than normal values and these levels continued during the course of pregnancy. At 18 gestational weeks, a transabdominal 2D ultrasonography detected a gestational age appropriate fetus that died *in-utero*. The fetus had an alobar holoprosencephalic brain (Fig. 1A) and decision was made to terminate the pregnancy. Subsequently, a *post mortem* examination disclosed findings of a male holoprosecenphalic fetus with a body mass of 26.8 grams, body length of 13 cm, occipito-frontal-circumference of 6 cm, cranio-caudal length of 8 cm, thoracic circumference of 6.5 cm, and abdominal circumference of 6 cm. The proband also showed classic facial features of the holoprosencephaly spectrum including cyclopia and absence of the nasal structures. The transabdominal 2D ultrasonography images (Fig. 1A) defined clearly the diagnosis of alobar holoprosencephaly with a monoventricular cavity with absence of the corpus callosum and a central mass that consists of fused thalamic nuclei (7). The diagnosis of early onset severe hydrocephalus could be excluded because external examination of the fetus identified classic facial features of the holoprosencephaly spectrum (cyclopia and absence of the nasal structures). Moreover, septo-optic dysplasia could be excluded because it is a possible differential diagnosis for lobar holoprosencephaly and not alobar holoprosencephaly (7). Dystrophic calcifications are also part of a calzo-cephalic process, which was absent (8).

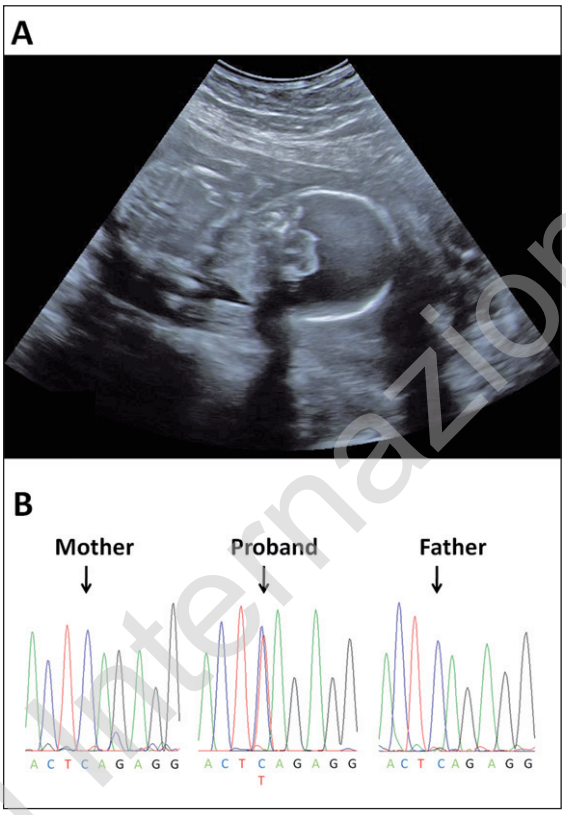

Figure 1. A) Transabdominal 2D ultrasonography at 18 gestational weeks showing a fetal brain manifesting alobar holoprosencephaly with a monoventricular cavity with absence of the corpus callosum and a central mass that consists of fused thalamic nuclei. B) A heterozygous c.298 C>T substitution mutation in the first exon of the *SHH* gene from the genomic DNA of the fetus, which is absent in both parents.

Methods

Since standard CTG band karyotyping on the mother, father and proband were normal, we undertook array CGH with the Aligent 44B array (Agilent Technologies, Mississauga, Ontario, Canada) which has 40,000 probes and an average spatial resolution of approximately 75 kb. 25 nanograms of total genomic DNA were used to detect chromosomal changes across the entire genome. The proband's DNA was labelled and co-hybridized against a reference DNA pool made of five normal male donors. The array slides were scanned with an Agilent microarray scanner and the data acquired using the Feature Extraction 8 software (Agilent). The CGH profiles were then created using the CGH-analytics 3.2 software (Agilent). Gains or losses of chromosomal content were flagged if present on at least two tailing probes to avoid false positives.

To complement aCGH analyses, we also used candidate genetic testing on the genomic DNA specimens from the parents and the proband on the four most commonly mutated genes in holoprosencephaly, namely, *SHH*, *ZIC2*, *SIX3* and *TGIF*. About 50 nanograms of genomic DNA from the fetus and both parents were utilized to amplify and sequence the coding regions of each exons and exon-intron boundaries of these four genes, using PCR primer pairs and conditions previously published (3). Polymorphic mutation studies utilized 50

nanograms of blood genomic DNA from 100 normal individuals, which were commercially obtained from the Human Random Control DNA panel (Sigma, Oakville, Ontario, Canada). All DNA sequence chromatograms were analyzed with the FinchTV v1.4 software, and sequences were aligned with the wildtype sequence using the FASTA program (www.ebi.ac.uk/Tools/fasta33/index.html) to identify mutations. Further bioinformatic analyses were undertaken on the wildtype and mutant protein sequences using proteomics and genomics tools available from Expasy (expasy.org) and the PolyPhen program (www.bork.embl-heidelberg.de/PolyPhen).

Results

Our analysis concluded that the proband is a novel sporadic case of alobar holoprosencephaly. Standard CTG band karyotyping did not disclose any plausible findings. Furthermore, using a whole genome screening approach to search for chromosomal gains or losses failed to detect two or more tailing probes. However, using instead a candidate mutational analyses approach, the fetus was identified with a heterozygous c.298 C>T substitution non-sense mutation in the first exon of the *SHH* gene (Fig. 1B), but no mutations were detected in the other HPE associated genes: *ZIC2*, *SIX3* and *TGIF*. This mutation was undetected in both parents, and in a panel of 100 normal control individuals (Fig. 1B), and likewise in the Single Nucleotide Polymorphic databases (www.humgen.nl/ SNP_databases.html). All these findings are suggestive of a sporadic and non-polymorphic mutation. This mutation is predicted to encode a truncated and non-functional protein (p.Gln100Ter). The protein truncates before the cholesterol mediated autoproteolytic cleavage site.

Discussion

In this study, we used combinations of classic CTG karotyping, with array CGH methods to undertake a genome-wide pre-screen for chromosome aberrations. Unfortunately, such screen failed to detect any chromosome imbalances or structural rearrangements. However, in conjunction with our screen by candidate gene sequence analyses, we were able to detect a mutation within the *SHH* gene in the proband. Within a diagnostic setting, our robust screening approach takes in practice up to two or three weeks to feasibly fulfill and within the context of having access to dedicated instrumentations and personnel.

A comparison of the findings from our case to the literature has identified three previous nonsyndromic HPE cases with heterozygous substitution mutations in the same codon 100 of the *SHH* gene. Two of such cases had the identical p.Gln100Ter non-sense mutation but were instead male subjects who inherited this mutation in an autosomal dominant manner from their non-penetrant parents (9). One sib had semilobar HPE with microcephaly, hypotelorism, midfacial hypoplasia and severe mental retardation, while the other one had alobar HPE with microcephaly, hypotelorism and cleft lip and palate (9). The third case was a male infant diagnosed with sporadic alobar HPE and died on the second day of life (10). This newborn infant had a c.300 G>C heterozygous substitution mutation resulting in a p.Gln100 His missense mutation in the SHH protein; a mutation predicted as a damaging variant (PolyPhen program).

Collectively, we report a new fetal case manifesting alobar holoprosencephaly with codon 100 of the *SHH* gene having a hotspot for loss-of-function mutations. All mutations detected in codon 100 of *SHH* thus far from this study and those previously (9,10) are predicted to be non-functional and contribute to the disease pathogenesis. Moreover, these mutations of codon 100 in *SHH* are identified in both sporadic and autosomal dominant inherited cases and with demonstrative evidence of variable expressivity and penetrance of the prosencephalon development deficiency spectrum. In summary, this study and others (11) reinforce the complexity of genotype-phenotype correlations through the molecular diagnostic process of holoprosencephalic fetuses. During the course of the pregnancy, fetal imaging coupled with standard and molecular cytogenetic, in conjunction with molecular genetic testing on suspected cases, still needs to continue, despite such spuriousness in variable penetrance and expressivity of the holoprosencephaly spectrum. The basis for these phenotypic differences is still largely unknown, but could be a consequence of several genetic and environmental factors during gestational development. Moreover, it must be considered that the possibility of additional mutations in yet to be discovered genes which contribute to the etiology of holoprosencephaly are potential confounding genetic factors that could explain such phenotypic heterogeneity.

Conclusion

With the availability of modern instrumentations and automated bioinformatics platforms, using combined whole genome-wide cytogenetic and candidate gene approaches are pivotal in accelerating the molecular diagnosis of prenatal anomalies within a short period, and hence fostering a closer link to achieving more accurate clinical diagnosis. Findings from this study also highlight the complexity of phenotypic heterogeneity among holoprosencephalic cases; a malformation disorder that most likely arises from having multiple genetic and environmental factors contributing to modifying the phenotype during gestational development.

Acknowledgements

We thank Mr. Huang for technical assistance and Dr. Kam Kamnasaran for helpful comments on this manuscript. Dr. Kamnasaran's work is supported by grants from the CHUQ foundation, Fondation des étoiles, Canadian Foundation for Innovation, Laval University Faculty of Medicine Foundation, Natural Sciences and Engineering Research Council, and Fonds de la recherche en

santé du Québec. Dr. Kamnasaran is a scholar from the Fonds de la recherche en santé du Québec.

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