ORIGINAL ARTICLE

Genotypic and phenotypic analysis of 396 individuals with mutations in Sonic Hedgehog

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ABSTRACT

Background Holoprosencephaly (HPE), the most common malformation of the human forebrain, may result from mutations in over 12 genes. Sonic Hedgehog (SHH) was the first such gene discovered; mutations in SHH remain the most common cause of non-chromosomal HPE. The severity spectrum is wide, ranging from incompatibility with extrauterine life to isolated midline facial differences.

Objective To characterise genetic and clinical findings in individuals with SHH mutations.

Methods Through the National Institutes of Health and collaborating centres, DNA from approximately 2000 individuals with HPE spectrum disorders were analysed for SHH variations. Clinical details were examined and combined with published cases.

Results This study describes 396 individuals, representing 157 unrelated kindreds, with SHH mutations; 141 (36%) have not been previously reported. SHH mutations more commonly resulted in non-HPE (64%) than frank HPE (36%), and non-HPE was more likely to have frank HPE than those with mutations in the other common HPE related genes (49% vs 35%, respectively; p=0.012). While mutations were significantly more common in the N-terminus than in the C-terminus (including accounting for the relative size of the coding regions, p=0.0001), no specific genotype-phenotype correlations could be established regarding mutation location.

Conclusions SHH mutations overall result in milder disease than mutations in other common HPE related genes. HPE is more frequent in individuals with truncating mutations, but clinical predictions at the individual level remain elusive.

INTRODUCTION

Holoprosencephaly (HPE), which results from failed or incomplete forebrain separation early in gestation, is the most common malformation of the human forebrain. HPE occurs in up to 1 in 250 conceptions, but in only approximately 1 in 10 000 liveborn infants due to the high proportion of intrauterine lethality.1 2 HPE is heterogeneous, and may result from large chromosomal imbalances, teratogenic agents, be found as one feature of an identifiable syndrome, or occur in a ‘non-syndromic’ context due to mutations in over 12 currently identified genes3 (reviewed in Solomon et al4).

Of the genes associated with HPE, Sonic Hedgehog (SHH), was the first identified.5 As with other HPE associated genes that were identified early, the involvement of SHH in human HPE was suggested by cytogenetic anomalies affecting chromosome 7q36, which contains the SHH locus.6 To date, mutations affecting SHH remain the single most common cause of non-chromosomal, non-syndromic HPE, accounting for approximately 12% of such cases7 (reviewed in Pineda-Alvarez et al8).

Most SHH mutations are family/individual-specific. Determining variant pathogenicity can be challenging, especially as functional assays are not available except in isolated research circumstances. Thus, assigning true ‘mutation’ status to a variant usually rests on the interpretation of an experienced molecular geneticist, and is based upon inheritance patterns, clinical features, and specific variant characteristics.7

As with other genes associated with non-chromosomal, non-syndromic HPE, SHH mutations result in an autosomal, dominantly inherited condition with apparently (we use the word ‘apparently’) many would agree that clinicians who are highly familiar with HPE and the range of manifestations in mutation-positive individuals would recognise subtle signs of mutation status in virtually all affected
carriers) incomplete penetrance and highly variable expressivity. \(^4\) HPE is categorised neuroanatomically by the degree of forebrain separation into alobar (the most severe type), semilobar, lobar, middle interhemispheric variant, and septoceleptopic types. \(^5\) \(SHH\)

mutation ‘carriers’ have been described as clinically unaffected by severe sequelae of disease, and are frequently referred to as having ‘microform’ HPE. Strictly, individuals with microform HPE should not be termed mutation carriers: they display subtle midline anomalies on physical examination, such as midface hypoplasia, hypotelorism, a flat or sharp nasal bridge, or a single maxillary central incisor, but will often not have detectable neuroanatomical anomalies or neurocognitive disturbances. \(^4\) In many families, more individuals will be affected with microform than with frank HPE. In these families, mutation status may be suspected upon the recognition of a severely affected individual. \(^4\) \(10\)–\(12\)

Through comparisons with cohorts of patients with mutations in the known HPE associated genes, some rudimentary intergenic genotype–phenotype correlations have been suggested. These include a preponderance of microform HPE resulting from mutations in \(SHH\), a specific facial phenotype in patients with \(ZIC2\) mutations, more severe types of HPE in patients with \(SIX3\) mutations, and an overrepresentation of renal tract anomalies in patients with mutations in \(SHH\) or \(ZIC2\). However, no intragenic genotype–phenotype correlations have been established. \(^4\) \(11\)–\(13\) \(14\)

In order to describe all known patients with mutations in \(SHH\) associated with HPE spectrum anomalies, we formed an international collaboration and collected data from the world’s largest HPE related molecular diagnostic centres. We describe 396 individuals, representing 157 unrelated kindreds, with mutations in \(SHH\). In addition to outlining clinical and molecular findings, we focus on attempts to predict phenotypic severity based on genotypic data.

**METHODS**

Patients were ascertained retrospectively through their respective institute review board approved research protocols, with appropriate consent, and through molecular laboratories (without identifying demographic data in this latter instance). Clinical details were supplied by the referring clinicians: requested items included completion of a standardised checklist, as well as materials such as a genetics consultation note, photographs, neuroimages, and other records, though the available information was highly variable. For patients identified through testing at the National Institutes of Health, sequencing was performed using previously published methodology. Published cases were obtained through a PubMed/Medline search, using the following search terms: Sonic Hedgehog, \(SHH\), holoprosencephaly, HPE (see supplementary table 1).

For specific portions of the presented analysis, we attempted to be conservative and not analyse variants of unknown significance along with ‘true’ pathogenic mutations. To do this, we intentionally excluded variants of unknown significance as defined on the basis of previous studies. \(^15\)–\(18\) In addition to literature based queries describing results of functional assays and analysis, variant pathogenicity in unclear instances was further investigated through software based prediction (Polyphen2), \(^19\) such that variants of possible but unproven pathogenicity according to the published literature that were then analysed as being unlikely to be pathogenic by software based prediction were excluded from analysis.

Statistical comparisons were made using \(\chi^2\) or Fisher’s exact test, where appropriate.

**RESULTS**

We describe 396 individuals (from 157 independent kindreds) with mutations in \(SHH\) (figure 1). One hundred and forty-one (36%) of these individuals have not been previously reported in the medical literature. Reports in the medical literature were ascertained from 1988 through the present (some early reported individuals were later found to have \(SHH\) mutations). The clinical features observed in these individuals comprise the entire phenotypic spectrum, from neuroanatomical anomalies incompatible with extrauterine life to isolated, extremely subtle
midline facial anomalies and reports of individuals who were described to be clinically 'unaffected'. Full data are available in supplementary table 1.

There was not a statistically significant gender disproportion in probands or total individuals. Of the 396 total individuals, gender was known in 359: of the 396, 185 (47%) were male and 174 (44%) were of unknown gender. One hundred and thirty-four of the 157 probands had known gender: 71 (45%) were male and 63 (40%) were female, while 23 (15%) of 157 probands were of unknown gender. The higher proportion of unknown proband gender largely results from an overrepresentation of severe HPE in probands compared to relatives (eg, due to a pregnancy loss with unknown fetal gender).

Of kindreds with a molecularly identified mutation in the proband, 98/157 (62%) had multiple affected relatives; however, familial testing was not always available. Of the 250 mutation carriers for whom inheritance was known, 158 (63%) had maternal inheritance, 80 (32%) had paternal inheritance, 25 (10%) of mutations were de novo, and 7 (3%) (including individuals from two different families) were identified as having germline mosaicism (most likely), as neither parent was found to have the mutation on peripheral blood testing, and paternity testing confirmed parentage. The high proportion of maternally inherited mutations may be secondary to increased maternal testing, though a more specific calculation regarding this is not possible, as details regarding which parents were tested are not uniformly available.

**MUTATIONS**

Among the 157 families, 141 unique SHH mutations were identified (figure 2). The largest proportion of variants (92/141, 66%) were missense, though 17/92 missense variants were considered to be variants of unknown significance (see Methods). Other mutation types included nonsense (21/141, 15%), frameshift (17/141, 12%), in-frame deletions or insertions (9/141, 6%), though 2/9 of these in-frame deletion/insertion variants were considered to be variants of unknown significance, and splice site mutations (2/141, 1%).

Of the 157 families, 14 different variants were each found in two apparently unrelated kindreds, and one mutation was found in three apparently unrelated kindreds. Of these non-unique mutations, three were nonsense mutations and four were missense mutations in the SHH-N domain, and two were nonsense and six were missense mutations in the SHH-C domain.

One proband had two variants in SHH (c.327C→T and c.528G→A), but the phase is unknown. Two probands had additional variants in CAS1, one had an additional variant in ZIC2, one had an additional variant in SIX3, and one had an additional variant in TGIF. The pathogenicity status of these non-SHH variants is not known, however, and it is entirely possible that these simply represent rare, non-pathogenic alleles.

In order to conduct a conservative analysis, after excluding possible variants of unknown significance (see Methods) 357 individuals with variants remained, comprising 123 unique variants. Of those variants, 73 variants occurred in the N-terminus and 50 in the C-terminus; there was a statistically significant difference in that mutations were more common in the N-terminus when compared to the expected ratio according to the number of bases in each region ($\chi^2=14.69, p=0.00010$). These differences remained significant when considering each specific type of mutation individually: single nucleotide substitutions, including those predicted to result in missense and nonsense mutations ($\chi^2=8.46, p=0.0036$) and frameshift substitutions ($\chi^2=11.90, p=0.00060$).

**Figure 2** (A) Distribution and types of variants, as well as severity of clinical manifestations are shown with the SHH predicted protein. (B) Individuals with frank HPE are shown below the predicted protein (with closed symbols), while individuals without evidence for neuroanatomical anomalies are shown above the predicted protein. As the figure illustrates, while overall trends may be analysed regarding genotype-phenotype correlations, individual level predictions remain elusive. HPE, holoprosencephaly; SHH, Sonic Hedgehog.
Genotype-phenotype correlations

CLINICAL FEATURES
Of the 157 probands, 83 (53%) had frank HPE. Of the probands, 27 (17%) had alobar, 36 (22%) had semilobar, 8 (5%) had lobar, and 12 (8%) had unknown type of HPE. Forty-four of 157 (28%) did not have HPE (were considered to be microform, or not HPE unknown) and 30 (19%) of them were unknown in terms of HPE affectedness. Of the relatives of probands, 49/239 (21%) had frank HPE. Of the relatives of probands, 15 (6%) had alobar, 5 (2%) had semilobar, 3 (1%) had lobar, and 26 (11%) had unknown type of HPE, and 174/239 (73%) did not have HPE (were categorised as ‘unaffected’ or microform). Sixteen (7%) of the mutation positive relatives were unknown with respect to HPE affectedness. Table 1 shows the distribution of the types of HPE among all individuals with frank HPE. Table 2 shows the classification of HPE type among all individuals with mutations (probands and relatives).

Individuals with frank HPE were universally severely cognitively impaired, and microcephaly and hypotelorism were very commonly reported in those with available data (due to lack of uniform data, we were unable to calculate specific percentages of these features). Clinical data obtained from other reports show that the most common features commented on were (of note, some of these features may be underreported due to variable clinical data) single central maxillary incisor (21%), cleft lip and/or palate (20%), choanal atresia (7%), coloboma (5%), reported diabetes insipidus (DI) (3%), reported pituitary dysfunction (in addition to DI) (3%), proboscis (2%), cyclopia or synophthalmia (2%), and ptosis (1%). Additionally, 5% had genitourinary/renal abnormalities including hypoplastic penis, cryptorchidism, renal hypoplasia, hypospadias, and ambiguous genitalia. Two percent had cardiac abnormalities (including persistently patent ductus arteriosus, ventricular septal defect, atrial septal defect, tricuspid atresia, interrupted inferior vena cava, and situs ambiguous). Renal anomalies and cardiac defects are not considered to be a classic component of non-chromosomal, non-syndromic HPE spectrum, although a recent large case series described renal anomalies in several individuals with SHH mutations.14

GENOTYPE–PHENOTYPE ANALYSIS
Many kindreds displayed wide intrafamilial phenotypic variability. After excluding variants of unknown significance, we compared the phenotypic severity in patients with truncating mutations to those with missense or in-frame small deletions/insertions, with the hypothesis that, despite limitations due to overgeneralisation, truncating mutations cause more severe phenotypes than hypomorphetic alleles resulting from missense or in-frame mutations. Of the truncating mutations with known phenotype, 65 (49%) individuals had frank HPE and 65 (51%) did not have frank HPE. Of the non-truncating missense or small in-frame deletions/insertions in individuals with known phenotype, 74 (55%) had HPE and 138 (65%) did not have HPE. There was a statistically significant difference such that individuals with truncating mutations had HPE more frequently than individuals with non-truncating mutations (p=0.012 by two-tailed Fisher’s exact test).

To evaluate the potential correlation between mutation location and phenotypic severity, we analysed missense and in-frame deletions/insertions (truncating mutations were not included secondary to the possibility of nonsense mediated decay). Thirty-four individuals with HPE and 53 without HPE had N-terminus mutations. Forty individuals with HPE and 85 without HPE had C-terminus mutations. There was not a statistically significant difference (p=0.31 by two-tailed Fisher’s exact test) between the groups with respect to severity of phenotype and mutation location.

We compared the prevalence of HPE (117 versus non-HPE (204) in patients with SHH mutations with previously published cohorts of patients with intragenic mutations affecting the other most common HPE associated genes (ZIC2: 88 HPE, 13 non-HPE; SIX3: 59 HPE, 33 non-HPE; and TGIF: 6 HPE, 7 non-HPE).11 13 23 After correcting for multiple comparisons, there was a statistically significant overrepresentation of non-HPE in the SHH cohort versus the SIX3 and ZIC2 cohorts (p<0.0001 for each comparison by two-tailed Fisher’s exact test), while there was not a statistically significant difference compared to the TGIF cohort (p>0.5 by two-tailed Fisher’s exact test, likely related at least in part to a very small comparison cohort).

DISCUSSION
We present a large cohort of patients with SHH mutations. To our knowledge, this is the largest described cohort with mutations in a single HPE gene. As mutations in this gene were the first and most commonly associated with HPE, SHH mutations are often considered to result in ‘prototypical’ HPE.24 Indeed, many of the findings described here reinforce this, including the presence of large families with multiple affected members of varying severity, as well as de novo mutations in a minority of cases, which is overall similar to the pattern seen in mutations in SIX3,11 but very different to that due to ZIC2 mutations.13 Despite the establishment of SHH as being associated with HPE over 15 years ago, there is still no firm explanation for the widely variable expressivity observed in these families, though plausible explanations typically involve multiple interacting genetic and environmental factors superimposed on a severely deleterious mutation. In virtually every case in which multiple mutations were purported, only one of the variants ultimately had any

Table 1 Distribution of holoprosencephaly (HPE) types among individuals with frank HPE

<table>
<thead>
<tr>
<th>HPE type</th>
<th>Probands with SHH mutation, n (%)</th>
<th>Individuals with SHH mutation, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alobar</td>
<td>27 (33%)</td>
<td>42 (32%)</td>
</tr>
<tr>
<td>Semilobar</td>
<td>36 (43%)</td>
<td>41 (31%)</td>
</tr>
<tr>
<td>Lobar</td>
<td>8 (10%)</td>
<td>11 (8%)</td>
</tr>
<tr>
<td>HPE (type unknown)*</td>
<td>12 (14%)</td>
<td>38 (29%)</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>132</td>
</tr>
</tbody>
</table>

*HPE (type unknown): individuals identified with true HPE, which was not further categorised into specific type.

Table 2 Distribution of holoprosencephaly (HPE) among all individuals

<table>
<thead>
<tr>
<th>HPE type</th>
<th>Probands with SHH mutation, n (%)</th>
<th>Individuals with SHH mutation, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alobar</td>
<td>27 (17%)</td>
<td>42 (11%)</td>
</tr>
<tr>
<td>Semilobar</td>
<td>36 (23%)</td>
<td>41 (10%)</td>
</tr>
<tr>
<td>Lobar</td>
<td>8 (5%)</td>
<td>11 (3%)</td>
</tr>
<tr>
<td>HPE (type unknown)*</td>
<td>12 (8%)</td>
<td>38 (10%)</td>
</tr>
<tr>
<td>Microform</td>
<td>42 (27%)</td>
<td>167 (42%)</td>
</tr>
<tr>
<td>None</td>
<td>0 (0%)</td>
<td>29 (7%)</td>
</tr>
<tr>
<td>Not HPE (type unknown)†</td>
<td>2 (1%)</td>
<td>22 (5%)</td>
</tr>
<tr>
<td>Unknown (no data)‡</td>
<td>30 (19%)</td>
<td>46 (12%)</td>
</tr>
<tr>
<td>Total</td>
<td>157</td>
<td>396</td>
</tr>
</tbody>
</table>

*HPE (type unknown): individuals identified with true HPE, which was not further categorised into specific type.
†Not HPE (type unknown): individuals identified without HPE but not further described.
‡Unknown (no data): individuals without any clinical data available.
evidence for pathogenicity, and thus may represent ‘red herrings’ (reviewed in Wannasilp et al). In fact, recent statistical evidence bears out the observation that the presence of multiple modifiers of individually small effect offers a better model of causality rather than a few digenic mutations of major effect.

We found that there is a significantly higher proportion of HPE in individuals with a truncating mutation versus a non-truncating mutation. This is clearly a very inexact comparison, as some missense mutations will certainly be highly functionally significant, but the overall trend is informative. Further functional studies may help determine the relative activity of these variants in order to perform correlations with our phenotypic data.

Interestingly, in our cohort, we have identified some families with a relatively large number of severely affected individuals, as well as other families in which many members are only mildly affected (tables 3 and 4).

Specifically, four families have high proportions of members who are severely affected. They have varying mutations:

On a mutation per base level, we found that there are statistically significantly more mutations in the N-terminus of the gene compared to the C-terminus. Specifically, there are significantly more total mutations, more substitution mutations (missense and nonsense), and more frameshift mutations. The explanation for this is unclear. For example, it could be postulated that N-terminus mutations confer a more severe phenotype and that individuals with mutations in the C-terminus may go undiagnosed more frequently. This cannot be the only explanation, as there are individuals with severe phenotypes who have mutations in the 3’ end of the gene. Again, the importance of functional studies arises here in order to compare the pathogenicity of variants in the C-terminus and the impact of those variants on processing. In addition, we note that the available clinical data are not uniform, which could affect the results.

We found no difference in HPE severity based on mutation location when comparing N-terminus to C-terminus mutations. However, there was again a significant difference when examining the location of the types of mutations, with significantly fewer truncating mutations in the C-terminus. It is unclear why there are fewer truncating mutations in the C-terminus compared to non-truncating mutations.

As mentioned, the major limitation to this study hinges on a lack of uniform clinical and functional data, which is unfortunately inevitable, as these cases were collected over the course of more than two decades from laboratories and clinics from a number of different countries, as well as from numerous previously published reports. However, the advantage of a large cohort helps ameliorate some of these shortcomings, and allows important observations that may be helpful for clinicians and diagnostic laboratories involved with the care of affected patients. We can conclude that non-HPE is overall more common than frank HPE in individuals with SHH mutations. Further, compared to cohorts of patients with mutations in the other two most common HPE associated genes, mutations in SHH more frequently result in non-HPE compared to frank HPE. Individuals with truncating mutations are more likely to have frank HPE than those with non-truncating mutations. Importantly, however, within any specific family or for any single person (eg, as applies to a mutation positive fetus, which is a frequently encountered clinical question), a prediction of individual severity is not currently possible. While extra-neuroanatomical/related facial anomalies are rare, cardiac and genitourinary anomalies are found in a small percentage of patients, including those without frank HPE. The majority of mutations in SHH occur in the N-terminus compared to the C-terminus and specifically that there is a paucity of truncating mutations in the C-terminus.

In summary, despite the lack of firm answers regarding major questions related to the clinical expression of SHH mutations, the information presented here should prove valuable to both the clinician and researcher for the purposes of helping frame questions about the pathogenicity of newly discovered variants, as well as counselling affected patients and families.

### Table 3 Families with a relatively large number of severely affected individuals

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Amino acid change</th>
<th>Type of mutation</th>
<th>Location</th>
<th>Individuals with HPE</th>
<th>Individuals without HPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.9_12dup4</td>
<td>p.Arg6GlyfsX59</td>
<td>Frameshift</td>
<td>N-terminus</td>
<td>4 alobar; 2 HPE type 1</td>
<td>1 microform</td>
</tr>
<tr>
<td>c.136C→T</td>
<td>p.Gln46X</td>
<td>Nonsense</td>
<td>N-terminus</td>
<td>1 semilobar; 2 HPE type 2</td>
<td>2 microform</td>
</tr>
<tr>
<td>c.383G→A</td>
<td>p.Trp128X</td>
<td>Nonsense</td>
<td>N-terminus</td>
<td>3 alobar</td>
<td>1 microform</td>
</tr>
<tr>
<td>c.664G→A</td>
<td>p.Asp222Asn</td>
<td>Missense</td>
<td>C-terminus</td>
<td>2 semilobar; 7 HPE type 3</td>
<td>3 microform; 1 none; 1 not HPE unknown</td>
</tr>
</tbody>
</table>

HPE, holoprosencephaly.
Contributors All authors contributed to the concept, design, acquisition of data or analysis and interpretation of data. All authors contributed to drafting or revising the content and approved the final version. The authors would like to express their deepest gratitude to all the patients and families who have participated in their research on holoprosencephaly and related conditions. We would also like to express gratitude to Julia Fekes and Darryl Leja for their help with illustrations and to Dr Alexa Pena Vargas, Department of Paediatrics, Endocrinology and Diabetes, Women’s and Children’s Hospital and University of Adelaide, South Australia for her referral.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by National Human Genome Research Institute IRB.

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Data sharing statement All relevant data are included in this article. All other clinical information specific to patients is managed by their respective clinicians and/or our reference laboratories.

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Genotype-phenotype correlations

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