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The Mutational Spectrum of Holoprosencephaly-Associated Changes within the *SHH* Gene in Humans Predicts Loss-of-Function Through Either Key Structural Alterations of the Ligand or Its Altered Synthesis

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Abstract

Mutations within either the *SHH* gene or its related pathway components are the most common, and best understood, pathogenetic changes observed in holoprosencephaly patients; this fact is consistent with the essential functions of this gene during forebrain development and patterning. Here we summarize the nature and types of deleterious sequence alterations among over one hundred distinct mutations in the *SHH* gene (64 novel mutations) and compare these to over a dozen mutations in disease-related Hedgehog family members *IHH* and *DHH*. This combined structural analysis suggests that dysfunction of Hedgehog signaling in human forebrain development can occur through truncations or major structural changes to the signaling domain, SHH-N, as well as due to defects in the processing of the mature ligand from its pre-pro-precursor or defective post-translation bi-lipid modifications with palmitate and cholesterol

Keywords

holoprosencephaly; mutation spectrum; SHH; protein processing

INTRODUCTION

Alterations in the *SHH* gene (Roessler et al., 1996,1997a;Nanni et al., 1999; MIM# 600725; HPE3 #142945) attributable to chromosomal rearrangements (Roessler et al., 1997b), microdeletions (Lacbawan and Muenke, in preparation; Bendavid et al., 2005a,b), or more

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commonly point mutations account for at least 6-8% of heterozygous sequence-detectable holoprosencephaly (HPE) related genetic variation (Orioli et al., 2001; Dubourg et al., 2004, 2007). Additionally, in many cases the molecular alteration presents as a familial form, often including many generations, where its clinically relevant phenotypic spectrum is highly variable between mutation carriers. Clinical findings can extend from cyclopia, at the severe extreme, to less severe conditions that include several types of recognizable microforms such as the solitary median maxillary central incisor syndrome (SMMCI; see Nanni et al., 2001;Marini et al., 2003;Garavelli et al., 2004; reviewed in El-Jaick et al., 2007a), or even incomplete penetrance of obligate mutation carriers. Mutations have also been detected in related pathway factors such as *PTCH1* (Ming et al., 2001; MIM# 601309; HPE7, MIM# 610828), the transcription factor gene *GLI2* (Roessler et al., 2005; MIM# 165230; HPE9, MIM# 610829) and the putative ligand transporter *DISP1* (Roessler et al., 2009; MIM# 607502). *SHH* is presently one of four genes, which also include *SIX3* (Wallis et al., 1999;Domené et al., 2008; MIM# 603714; HPE2, MIM# 157170), *ZIC2* (Brown et al., 1998;Roessler et al., 2009; MIM# 603073; HPE5, MIM# 609637) and *TGIF* (Gripp et al., 2000;El-Jaick et al., 2007b; MIM# 602630; HPE4, MIM# 142946), routinely screened as part of the molecular evaluation of new sporadic or familial HPE or SMMCI cases (reviewed in Muenke and Beachy, 2001;Cohen, 2006;Dubourg et al., 2007). Consequently, a large collection of mutations is presently available for a structural analysis.

The vertebrate *hedgehog* (*hh*) family of genes actively function during major developmental steps including pattern formation and growth of the brain, neural tube, myotome, skeletal structures and limb bud, as well as during tissue specification and growth of a variety of organs by acting as context-dependent morphogens and/or mitogens during early development (Cordero et al., 2004; see recent reviews by Ingham 2008; Bürglin 2009). An additional role in somatic cancer is also well documented. All known hedgehog proteins are synthesized as pre-pro-proteins that during their traffic through the endoplasmic reticulum undergo the removal of a signal peptide, followed by autocatalytic cleavage into a 19Kd secreted molecule (e.g. SHH-N, with all known biological activity) and a 25 Kd intein-like residual component [e.g. SHH-C, with the intrinsic ability to cleave the precursor into two fragments (due to the activity of the intein-like Hint motif) and also to add cholesterol to the C-terminus of its partner fragment (facilitated by the sterol-recognition region, SRR); for a review, see Muenke and Beachy 2001; Porter et al., 1995, 1996]. The biological activity of hh-like ligands, such as the human SHH-N, is further enhanced by the addition of palmitate to its N-terminus by the substrate-specific enzyme Hedgehog acyltransferase (Pepinsky et al., 1998; HHAT; MIM# 605743). This bi-lipidated ligand is actively secreted from the cell with the participation of additional co-factors, such as DISP1, that themselves can be targets of mutation in HPE cases (Ma et al., 2002; Roessler et al., 2009). The gradient of these signaling factors that will develop in a target field is influenced by many additional factors including heparin-sulfate proteoglycans, the assembly of multimeric complexes of ligands (Goetz et al., 2006; Singh et al., 2008), as well as both positive (GAS1 and CDO/BOC) and negative modulators (enhanced expression of PTCH1 and HIP) of the cell surface binding and response of target cells (see reviews by Krauss 2007 and Ingham 2008).

Over the past decade, molecular defects have been identified in all three paralogous genes of the prototypical *Drosophila melanogaster* hedgehog gene (*hh*) typically present in higher animals, such as humans: *INDIAN HEDGEHOG (IHH* with autosomal dominant Brachydactyly A-1, BDA1; MIM# 112500 and the autosomal recessive disorder acrocapitalfemoral dysplasia, ACFD; MIM# 607778; Gao et al., 2001; McCready et al., 2002; Liu et al., 2006; Lodder et al., 2008; reviewed in Byrnes et al., 2009), *DESERT HEDGEHOG* (*DHH* with gonadal dysgenesis; MIM# 605423; Umehara et al., 2000; Canto et al., 2004, 2005) and *SONIC HEDGEHOG* (with HPE and related disorders, see above). Fortunately, the past decade has also coincided with an extensive and detailed analysis of the

biological effects and functional domains of the proto-typical hedgehog gene that can provide a foundation for predicting the effects of disease-related mutations (Hall et al., 1995, 1997; McLellan et al., 2008; Goetz et al., 2006; Singh et al., 2009; reviewed in Bürglin 2008). Based on these considerations it's now possible to show that essentially all key steps in the generation of a secreted hedgehog molecule are targets for mutation in a collective retrospective analysis of cases.

MATERIALS AND METHODS

Study population

At the NIH, we analyzed approximately 600 HPE patients (collectively comprising the entire spectrum of HPE brain malformations and prospectively collected over 17 years) for potential sequence variations in the *SHH* gene under our NHGRI approved brain research protocol and newly established CLIA laboratory. In addition, we also studied 125 unrelated individual normal controls obtained as anonymous samples from the Coriell Institute for Medical Research that matched the predominant Northern European ethnicity of our HPE cases. In the cases extracted from literature reports the nature of the mutation was known to us only through these published sources (cited in Table 1). Similarly, in Rennes, 500 HPE patients were analyzed, prospectively collected over 12 years at the Laboratoire de Génétique Moléculaire (Rennes, France); we also include anonymous instances of mutations in the *SHH* gene shared with us from prospective studies performed under CLIA standards by GeneDx (Gaithersburg, MD), or from investigators in Maastricht, the Netherlands, as well as from Regensburg, Germany.

Mutation screening, PCR amplification and DNA sequencing

A strategy for screening the *SHH* gene (available on request) has been modified from previously described ones (Roessler et al., 1996,1997a;Dubourg et al., 2004) using only four pairs of primers resulting in four amplicons instead of the previous six (1F1/1R1, 2F2/2R1, 3F1/3R1 and 3F3/3R3). Descriptions of mutations (Table 1) are based on the NM_000193.2 reference sequence. Guidelines for the naming of the sequence variants conform to the recommendations of the human nomenclature committee (www.hgvs.org/mutnomen) and were checked using the Mutalyzer software for the predicted effects on the reference gene (www.lovd.nl/mutalyzer/1.0.1/). Position +1 refers to the A position of the ATG initiation codon for that gene.

Our current approach for CLIA testing of *SHH* also differs slightly from previous reports and is available upon request. Amplification of human genomic DNA is performed in a 35 μl reaction volume, using 60-100 ng of DNA template, 50 μM each of deoxynucleotide triphosphate, 20 pmol of each primer, 3.5 μl of 10X PCR Amplification buffer (Invitrogen, CA), 1.75 μl 10X PCR Enhancer solution (Invitrogen, CA), 1.5mM MgSO4 (Invitrogen, CA) and 2.5U of AmpliTaq DNA polymerase (Roche, IN). All reactions were performed using a PTC-255 thermocycler (MJ Research, MA). Typical PCR cycling parameters were 95°C for 4 minutes followed by 30 cycles at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and a final extension step of 72°C for 7 minutes. One half of the PCR product was used for denaturing high-pressure liquid chromatography (dHPLC) analysis (Trangenomics WAVE™) and the remainder was retained for direct DNA sequencing. Amplicons displaying heterozygous profiles were purified using a high pure PCR purification kit (Roche, IN) and bi-directionally sequenced using the BigDye™ version 3.1 terminator cycle sequencing kit according to the manufacturer's protocol (Applied Biosystems, CA) on an ABI 3100 automated sequencer.

Statistical analysis

To determinate whether the distribution of observed and expected number of mutations within SHH-N or SHH-C were the same, a classic two-tailed χ^2 test was used. In order to detect domains (SHH-N vs. SHH-C) for which the observed number of mutations differed from the expected values, we conducted the following hypothesis test:

$$H_{0,j}: p_{O,j} = p_{E,j} \quad \text{vs.} \quad H_{1,j}: p_{O,j} \neq p_{E,j}$$

where $p_{O,j}$ and $p_{E,j}$ represent, respectively, the proportion of observed and expected mutations in the j domain (j = Identical, Similar, Related, Unrelated or Biologically Equivalent). Considering that the proportion of observed or expected mutations in the Biologically Equivalent (BE) was calculated as $p_{BE}=1 - p_U$ (U: Unrelated), testing $H_{0,j} : p_{O,BE} = p_{E,BE}$ is mathematically identical (but not biologically equivalent) to the test of $H_{0,j} : p_{O,U} = p_{E,U}$.

We used R 2.9.0 Patched (R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org) for the statistical analysis, the RODBC (Lapsley, M. and Ripley, B.D. 2009. RODBC: ODBC Database Access. http://cran.r-project.org/web/packages/RODBC/index.html) package to connect R and MS Excel®, and Tinn-R (Tinn-R Development Team 2004; http://www.sciviews.org/Tinn-R/ and http://sourceforge.net/projects/tinn-r) as editor for the R programs we wrote.

RESULTS

In order to derive insight into the important functions and domains of the SHH protein inferred from our mutational analysis, we have compiled into Table 1 all 121 of the known SHH variants detected exclusively among HPE cases and not in controls. When these alterations are aligned with other mutant Hedgehog proteins and annotated with the detailed structural and functional studies of the murine Shh protein, several important findings emerge from this compilation (broken down for convenience by mutation type in Figure 1A-C). Most of these types of variation have either been directly shown to reduce biological activity (Schell-Apacik et al., 2003;Traiffort et al., 2004;Maity et al., 2005;Goetz et al., 2006;Singh et al., 2009) or would be predicted to lose activity by analysis of the effects on key structural motifs of virtually translated protein products. However, the precise effects of individual mutations can rarely be predicted with certainty without detailed and often laborious functional analysis.

Evidence for mutations likely causing processing defects of the SHH protein

Since any observed mutation in SHH-C would not contribute directly to the structure of the SHH-N ligand itself, and yet full biological activity is dependent on the success of many successive processing steps, it was originally postulated that defective maturation of the protein could the basis for the pathogenicity of some of these mutations (Roessler et al., 1997a; Hall et al., 1997). We can now affirm that mutations in HPE cases are non-randomly clustered, and commonly occur in precisely those key elements that contribute to successful processing, the absence of which is known to dramatically reduce protein function.

Interestingly, in addition to the single example of elimination of the initiator methionine of DHH (Umehara et al., 2000) there are two additional examples of missense changes detected in the signal peptide (p.Arg6Thr and p.Leu17Pro) of SHH that might interfere with entry into the endoplasmic reticulum; although neither variant has been analyzed functionally in transfected cells, the p.Leu17Pro variant segregates among affected individuals in a large pedigree. Similarly, a cluster of mutations are detected in the highly conserved CGPGR motif at the amino-terminus implicated in both the addition of palmitate (e.g. p.Pro26Leu and

p.Gly27Ala) and the formation a multimeric high potency forms (Goetz et al., 2006; Singh et al., 2008). Experimental mutation of these or similar residues in the rat Shh protein alters its ability to be lipid modified, secreted and/or form full active multimer forms (Goetz et al., 2006, see also Fig.2). Another cluster of missense alterations and precise deletions eliminate the cholesterol addition site (p.Gly197; detailed in Fig. 1, 2) at the end of the ligand domain: p.196_200del, p.Gly196GlufsX20, p.Gly196Glu, p.Gly197Val, p.Cys198Phe and p.Cys198Ser. Thus, the mutational spectrum observed in patients directs our attention to essential motifs independently shown to be necessary for the production of a bioactive molecule.

The mutations observed in both the Hint module and SRR motifs are biologically plausible as loss-of-function alterations based on site-directed mutagenesis studies (Fig. 2, see also Hall et al., 1997) and are consistent with our general observation that they occur preferentially within conserved residues important for this structure (see chi squared analysis, Table 2). For example, the positions p.Asp222, p.Val224, p.Phe241, p.263_269del, p.Thr267, p.Leu271 and p.Ile354 correspond to positions that are highly conserved residues among intein-like elements across many phyla and it is statistically significant that they are also sites of mutation in HPE subjects (see also Traiffort et al., 2004 for examples of confirmed deleterious mutations in SHH-C; see Canto et al., 2004 for a similar mutation in DHH). This pattern of mutation within largely conserved residues applies to the majority of the mutations observed in the Hint motif (p.Leu218Pro, p.Ser236Asn, p.Ile255Asn, p.Val332Ala, p.Ala346Val, p.Pro347Gln, p.Pro347Arg, p.Pro347Leu, p.Ser362Leu, p.Cys363Tyr, p.Tyr364Cys). In fact, a similarly strong mutational signature is also observed in the SRR region that functions to bind cholesterol and uses it as a nucleophile in the autoprocessing reaction: p.Ala373Thr, p.His374Arg, p.Ala376Asp, p.Phe377Ser, p.378_380del, p.Arg381Pro, p.Leu382Pro and p.Ala383Thr. Note that the last position in this cluster is also the site of a nonsense mutation in DHH.

Typical frameshift and truncation mutations

Nearly a quarter of all of the mutations detected (29/121) are frameshifts or truncations incompatible with a complete protein. All but one (pMet457ArgfsX18) would be expected to eliminate the intein-like Hint motif necessary for autocatalytic processing and would be considered as likely biological null alleles.

Missense alterations of both SHH-N and SHH-C occur in functionally defined domains

The distribution of HPE-associated mutations in the mature SHH-N domain continues to follow the pattern of conservation established for SHH-C (Table 2) although, counter-intuitively, the level of significance is less because of the overall high degree of conserved motifs; in addition, those mutations tend to be observed within centrally-located conserved residues thought to be important in ligand-receptor interactions or similar structurally relevant modules. For example, p.Glu176_Lys178del removes residues shown to be essential for the binding between Shh and the Fibronectin3 motif of the CDO receptor (McClellan et al., 2008). In general, mutations in IHH and DHH also occur in centrally located conserved residues (see Fig. 2; Byrnes et al., 2009); furthermore, it is unlikely to be a co-incidence that mutation of SHH-N residues p.Thr150 is also a site for mutation in IHH.

Unclassified mutations

Some variants do not fall into any clear category, other than that they are not generally conserved from flies to higher vertebrates: such as p.Gly290Asp, p.Gly296Ala, p.Ala391Thr, p.402_409del, p.405_409del, p.Gly411dup, p.Thr416Ala, p.Pro424Ala and p.Gly456Arg (Nanni et al., 1999; Schimmenti et al., 2001). It is probable that there will be instances of highly conserved residues being non-significant when functional tests are eventually performed (see Maity et al., 2005), as well as instances where seemingly un-obvious mutations have profound

effects. For example, our alignment analysis fails to take into account human-specific features evolved in the primate lineage (Dorus et al., 2006). These authors note that SHH-C has undergone accelerated evolution within primates and the non-random acquisition of multiple serines and threonines that may serve as sites of post-translational modification. For example, the p.Gly290Asp mutation might affect the phosporylation state of p.Ser291 or the deletions p.402_409del or p.405_409del the postulated phosphorylation of p.Ser401. Additional studies would be needed to test this hypothesis. Hence, the best way for a clinician to establish the likelihood of a mutation in these genes being clinically relevant would be to carefully examine the family history and mutation status of additional family members.

DISCUSSION

Holoprosencephaly is the most common structural malformation of the developing forebrain in humans and its causation includes genetic and environmental components typical for a complex trait that must integrate both types of factors (Muenke and Beachy, 2001; Roessler et al., 2001, 2003, 2008; Cohen, 2006; Dubourg et al., 2007; Krauss 2007; Monuki 2007). Its genetic heterogeneity is supported by the fact that nearly 75% of cases are wild-type in their DNA sequence of the coding regions of the nine currently known HPE genes in patients with normal chromosomes. Its characteristic variable expressivity adds additional support for a model of combinatorial interactions among multiple factors (Ming and Muenke, 2002). Although much more needs to be elucidated regarding the potential roles of additional genes, we are beginning to see the emergence of a network of factors in the mouse that converge on the expression of Sonic hedgehog as a common final pathway in HPE etiology (Jeong et al., 2008; Geng et al., 2008; Warr et al., 2008).

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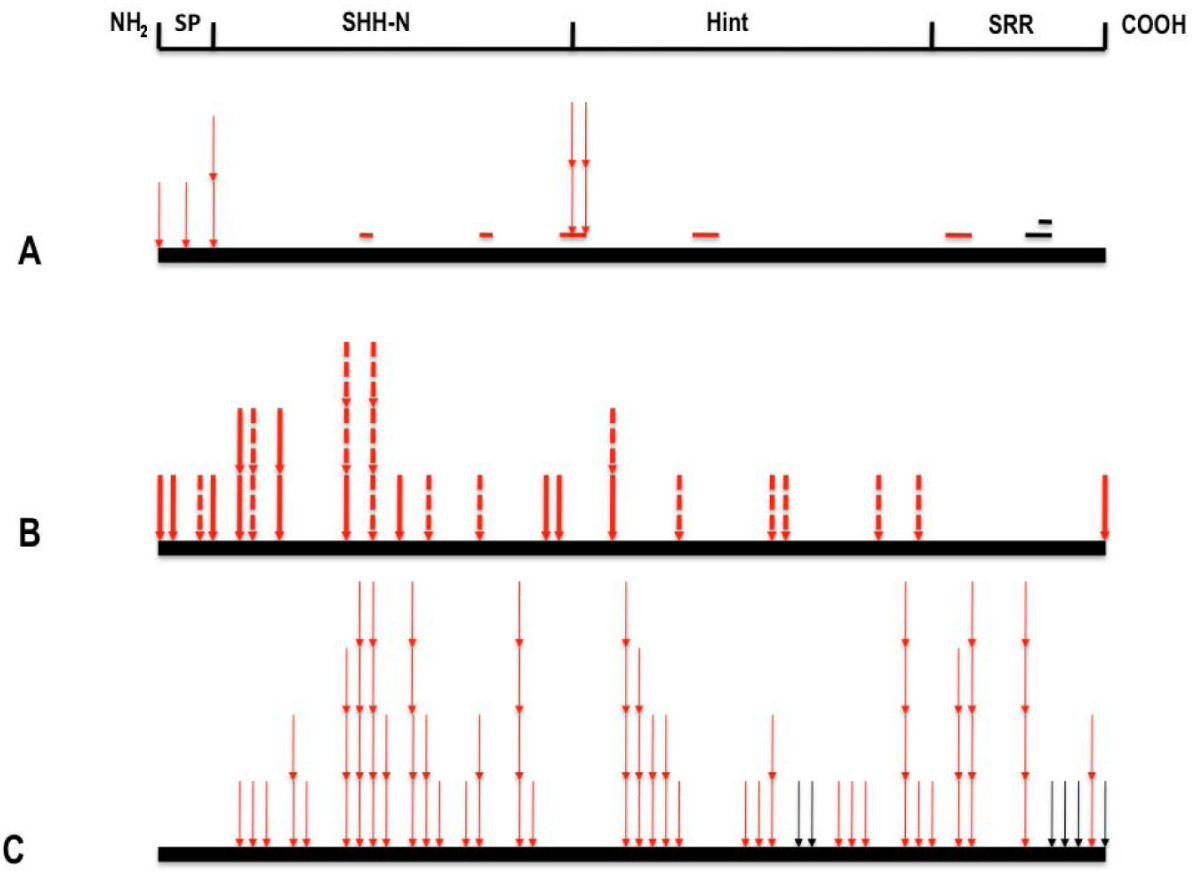

Figure 1.

Compilation of all of the mutations affecting the two principal SHH domains sorted by their position and category. **A**: deletions or insertions that retain frame occur most commonly in critical regions (red colored bars) but also non-conserved regions of unknown importance (black colored bars; see also Fig. 2); selected missense alterations (thin arrows) that occur in the signal peptide, palmitate addition region, cholesterol addition site are presented here to imply their likely effect on processing. **B**: nonsense and frameshift mutations truncate SHH-N or affect domains required for protein intein-like splicing or cholesterol addition. **C**: missense alterations (thin arrows) occur equally in both domains; however, the distribution of mutations is non-random and preferentially occurs within conserved domains (see Table 2).

```
ref|NP_000184.1|          -----------MLLLARCLLLVLVSSLLVCSGLACGPGR-GFG-KRRHPK  37   SHH
ref|NP_033196.1|          ----------MLLLLARCFLVILASSLLVCPGLACGPGR-GFG-KRRHPK  38   Shh
ref|NP_002172.2|          -------MSPARLRPRLHFCLVLLLLLVVPAAWGCGPGR-VVGSRRRPPR  42   IHH
ref|NP_066382.1|          ------------MALLTNLLPLCCLALLALPAQSCGPGRGPVGRRRYARK  38   DHH
ref|NP_001034065.1        MRHIAHTQRCLSRLTSLVALLLIVLPMVFSPAHSCGPGR-GLG--RHRAR  97   hh
                                                :    ::  .. .***** .*  *   :

ref|NP_000184.1|          KLTPLAYKQFIPNVAEKTLGASGRYEGKISRNSERFKELTPNYNPDIIFK  87   SHH
ref|NP_033196.1|          KLTPLAYKQFIPNVAEKTLGASGRYEGKITRNSERFKELTPNYNPDIIFK  88   Shh
ref|NP_002172.2|          KLVPLAYKQFSPNVPEKTLGASGRYEGKIARSSERFKELTPNYNPDIIFK  92   IHH
ref|NP_066382.1|          QLVPLLYKQFVPGVPERTLGASGPAEGRVARGSERFRDLVPNYNPDIIFK  88   DHH
ref|NP_001034065.1        NLYPLVLKQTIPNLSEYTNSASGPLEGVIRRDSPKFKDLVPNYNRDILFR 147   hh
                          :* **  **  *.:.* * .***  ** : *.* :*::*.**** **:*:

ref|NP_000184.1|          DEENTGADRLMTQRCKDKLNALAISVMNQWPGVKLRVTEGWDEDGHHSEE 137   SHH
ref|NP_033196.1|          DEENTGADRLMTQRCKDKLNALAISVMNQWPGVKLRVTEGWDEDGHHSEE 138   Shh
ref|NP_002172.2|          DEENTGADRLMTQRCKDRLNSLAISVMNQWPGVKLRVTEGWDEDGHHSEE 142   IHH
ref|NP_066382.1|          DEENSGADRLMTERCKERVNALAIAVMNMWPGVRLRVTEGWDEDGHHAQD 138   DHH
ref|NP_001034065.1        DEEGTGADRLMSKRCKEKLNVLAYSVMNEWPGIRLLVTESWDEDYHHGQE 197   hh
                          ***.:******::***:::* ** :*** ***::* ***.**** **.::

ref|NP_000184.1|          SLHYEGRAVDITTSDRDRSKYGMLARLAVEAGFDWVYYESKAHIHCSVKA 187   SHH
ref|NP_033196.1|          SLHYEGRAVDITTSDRDRSKYGMLARLAVEAGFDWVYYESKAHIHCSVKA 188   Shh
ref|NP_002172.2|          SLHYEGRAVDITTSDRDRNKYGLLARLAVEAGFDWVYYESKAHVHCSVKS 192   IHH
ref|NP_066382.1|          SLHYEGRALDITTSDRDRNKYGLLARLAVEAGFDWVYYESRNHVHVSVKA 188   DHH
ref|NP_001034065.1        SLHYEGRAVTIATSDRDQSKYGMLARLAVEAGFDWVSYVSRRHIYCSVKS 247   hh
                          ********: *:*****:.***:********** * *: *:: ***:

ref|NP_000184.1|          ENSVAAKSGGCFPGSATVHLEQGGTKLVKDLSPGDRVLAADDQGRLLYSD 237   SHH
ref|NP_033196.1|          ENSVAAKSGGCFPGSATVHLEQGGTKLVKDLRPGDRVLAADDQGRLLYSD 238   Shh
ref|NP_002172.2|          EHSAAAKTGGCFPAGAQVRLESGARVALSAVRPGDRVLAMGEDGSPTFSD 242   IHH
ref|NP_066382.1|          DNSLAVRAGGCFPGNATVRLWSGERKGLRELHRGDWVLAADASGRVVPTP 238   DHH
ref|NP_001034065.1        DSSISSHVHGCFTPESTALLESGVRKPLGELSIGDRVLSMTANGQAVYSE 297   hh
                          : * : :  ***.  : . * .*    :  :  ** **:   .*    :
```

Cholesterol addition site

Figure 2. A detailed annotation of known mutations within closely related Hedgehog proteins

A clustalW2 based (http://www.ebi.ac.uk/Tools/clustalw2/) single aminoacid code alignment of SHH, murine Shh, IHH, DHH and hh is presented. Protein changes are annotated by color and font. For example, HPE or SCMMI alterations are red mutations: normal font are typical missense, red underlined positions are in-frame deleted residues in functionally important motifs, while in-frame deletions or insertions of unknown effect are depicted as black lines above the alignment (p.402_409del and p.405_409del) or as a triangle (i.e. p.Gly411dup). For the mouse Shh alignment we use orange residues with normal font as synthetically engineered point mutations (see Hall et al., 1997; Goetz et al., 2006; Singh et al. 2008), and surface residues implicated in Shh-N-CDOFn3 (ligand-receptor) binding (McLellan et al. 2008) are underlined with a saw-tooth font. In contrast, residues implicated in binding to the fly paralog, Ihog, are orange and underlined. These authors also identified residues that co-ordinate with calcium

and promote receptor binding that we present as orange and *italic*. In addition, IHH (Brachydactyly A-1 or acrofemoralcapital dysplasia; turquoise mutations), DHH (gonadal dysgenesis; green mutations) are presented and compared directly to the *Drosophila* hh reference sequence. Each paralog encodes a signal peptide shown as underlined normal font (the signal peptide of hh is not shown) and a Sterol Recognition Region (SSR) that is both underlined and italicized. Note that key motifs necessary for hh auto-processing [e.g. the Shh Hint motif (p.258_408) consists of functionally conserved core aminoacids that are show in purple and underlined; a subset of these have been shown to be functionally essential and are double underlined: D303, T326 and H329 (Hall et al. 1997).

Table 1

SHH mutations identified in this study. Novel variants are in bold type.

Subject	cDNA	protein	function	Reference
1	c.9_12dup	p.Arg6GlyfsX59	frameshift	Nanni 1999
2	c.17G>C	p.Arg6Thr	Signal peptide	Dubourg 2004
3	c.38_45del	p.Val13AlafsX48	frameshift	Nanni 1999; GeneDx confirmed
4	c.50T>C	p.Leu17Pro	Signal peptide	Kato 2000
5	c.72C>A	p.Cys24X	Palmitoylation site	Dubourg 2004; NIH new
6	**c.77C>T**	**p.Pro26Leu**	**Palmitoylation site and multimerization**	**GeneDx new; see** Goetz 2006
7	c.80G>C	p.Gly27Ala	Palmitoylation site and multimerization	Hehr 2004
8	**c.87delG**	**p.Gly29fsX11**	**frameshift**	**Rennes new**
9	c.91G>A	p.Gly31Arg	SHH-N	Roessler 1996
10	**c.116T>C**	**p.Leu39Pro**	**SHH-N**	**GeneDX new**
11	**c.108_109delinsG**	**p.Lys38SerfsX2**	**frameshift**	**NIH new**
12	**c.119_120delCC**	**p.Pro41PhefsX22**	**frameshift**	**Rennes new**
13	**c.136C>T**	**p.Gln46X**	**truncation**	**NIH new**
14	**c.148_166del**	**p.Asn50X**	**truncation**	**Maastrict new**
15	**c.157G>A**	**p.Glu53Lys**	**SHH-N**	**Rennes new**
16	**c.210dupC**	**p.Glu71ArgfsX4**	**frameshift**	**GeneDx new**
		p.Gly290Asp	SHH-C	See 89
17	c.211delG	p.Glu71SerfsX27	frameshift	Dubourg 2004
18	**c.248A>T**	**p.Asp83Val**	**SHH-N**	**Rennes new**
19	c.250A>T	p.Ile84Phe	SHH-N	NIH new
20	c.263A>T	p.Asp88Val	SHH-N	Nanni 1999: Heussler 2002; Regensburg new
21	**c.298delC**	**p.Gln100ArgfsX7**	**frameshift**	**GeneDx new**
22	c.298C>T	p.Gln100X	truncation	Roessler 1996; Rennes new
23	c.300G>C	p.Gln100His	SHH-N	Dubourg 2004; Bertolacini 2009
24	**c.304T>C**	**p.Cys102Arg**	**SHH-N**	**Maastricht new**
25	**c.305G>A**	**p.Cys102Tyr**	**SHH-N**	**Rennes new**
26	c.316_321del	p.Leu106_Asn107del	deletion	Dubourg 2004
27	c.313A>T	p.Lys105X	truncation	Roessler 1996
28	**c.327G>T**	**p.Leu109Phe**	**SHH-N**	**GeneDx new**
29	**c.328G>A**	**p.Ala110Thr**	**SHH-N**	**GeneDx new**
30	c.329C>A	p.Ala110Asp	SHH-N	Dubourg 2004
31	c.331A>T	p.Ile111Phe	SHH-N	Nanni 2001
32	c.332T>A	p.Ileu111Asn	SHH-N	El-Jaick 2005
33	c.345C> A	p.Asn115Lys	SHH-N	Nanni 1999
34	c.349T>G	p.Trp117Gly	SHH-N	Roessler 1996
35	c.349T>C	p.Trp117Arg	SHH-N	Roessler 1996
36	**c.355G>T**	**p.Gly119X**	**truncation**	**NIH new**
37	**c.370G>A**	**p.Val124Met**	**SHH-N**	**GeneDx new**
38	c.383G>A	p.Trp128X	truncation	Marini 2003
39	c.388G>T	p.Glu130X	truncation	Dubourg 2004; NIH new
40	c.406G>A	p.Glu136Lys	SHH-N	Bertolacini 2009
41	**c.415_416del**	**p.Leu139AlafsX13**	**frameshift**	**NIH new**
42	c.419A>C	p.His140Pro	SHH-N	Orioli 2001
43	c.420C>G	p.His140Gln	SHH-N	Ribiero 2005
44	**c.428G>A**	**p.Gly143Asp**	**SHH-N**	**NIH new**
45	**c.431G>C**	**p.Arg144Pro**	**SHH-N**	**GeneDx new**
46	**c.439G>A**	**p.Asp147Asn**	**SHH-N**	**GeneDx new**
47	c.449C>G	p.Thr150Arg	SHH-N	Dubourg 2004; NIH new
48	**c.449C>A**	**p.Thr150Lys**	**SHH-N**	**Rennes new**
49	**c.468C>A**	**p.Ser156Arg**	**SHH-N**	**NIH new**
50	c.474C>G	p.Tyr158X	truncation	Dubourg 2004
51	**c.509T>G**	**p.Phe170Cys**	**SHH-N**	**NIH new**
52	**c.511G>C**	**p.Asp171His**	**SHH-N**	**Rennes new**

Subject	cDNA	protein	function	Reference
53	**c.525C>G**	**p.Tyr175X**	**SHH-N**	**Regensburg new**
54	c.526_534del	p.Glu176_Lys178del	deletion	Dubourg 2004
55	**c.547T>C**	**p.Cys183Arg**	**SHH-N**	**NIH new**
56	c.548G>T	p.Cys183Phe	SHH-N	Orioli 2001
57	**c.548G>A**	**p.Cys183Tyr**	**SHH-N**	**Regensburg new**
58	**c.551C>T**	**p.Ser184Leu**	**SHH-N**	**NIH new**
59	c.562G>C	p.Glu188Gln	SHH-N	Dubourg 2004
60	**c.562+1G>A**	**Splice donor**	**frameshift**	**NIH new**
61	**c.584_598del**	**p.196_200del**	**Cholesterol addition site**	**NIH new; GeneDx confirmed**
62	**c.587delG**	**p.Gly196GlufsX20**	**Cholesterol addition site**	**Rennes new**
63	**c.587G>A**	**p.Gly196Glu**	**Cholesterol addition site**	**Rennes new**
64	**c.590G>T**	**p.Gly197Val**	**Cholesterol addition site**	**NIH new**
65	**c.592T>A**	**p.Cys198Ser**	**Cholesterol addition site**	**Rennes new**
66	c.593G>A	p.Cys198Phe	Cholesterol addition site	Bertolacini 2009
67	**c.624_625delinsTT**	**p.Glu208AspfsX1**	**frameshift**	**GeneDx new**
68	c.625C>T	p.Gln209X	truncation	Nanni 1999
69	c.653T>C	p.Leu218Pro	SHH-C	Richieri_Costa 2006
70	c.664G>A	p.Asp222Asn	SHH-C	Dubourg 2004
71	c.671T>A	p.Val224Glu	SHH-C	Roessler 1997
72	c.676G>A	p.Ala226Thr	SHH-C	Roessler 1997
73	**c.692G>T**	**p.Gly231Val**	**SHH-C**	**NIH new**
74	**c.694C>G**	**p.Arg232Gly**	**SHH-C**	**GeneDx new**
75	**c.701T>C**	**p.Leu234Pro**	**SHH-C**	**GeneDx new**
76	**c.707G>A**	**p.Ser236Asn**	**SHH-C**	**Rennes new**
77	c.708C>A	p.Ser236Arg	SHH-C	Nanni 1999
78	**c.721T>C**	**p.Phe241Val**	**SHH-C**	**Rennes new**
79	**c.723C>G**	**p.Phe241Leu**	**SHH-C**	**NIH new**
80	**c.764T>A**	**p.Ile255Asn**	**SHH-C**	**NIH new**
81	c.766G>T	p.Glu256X	truncation	Nanni 1999; GeneDx confirmed
82	c.787_807del	p.Arg263_Ala269del	deletion	Roessler 1997a
83	c.800C>T	p.Thr267Ile	SHH-C	Hehr 2004
84	c.812T>C	p.Leu271Pro	SHH-C	Dubourg 2004
85	**c.824C>A**	**p.Ala275Glu**	**SHH-C**	**GeneDx new**
86	**c.839C>G**	**p.Ser280Trp**	**SHH-C**	**Regensburg new**
87	c.839C>A	p.Ser280X	truncation	Richieri-Costa 2006
88	c.850G>T	p.Glu284X	truncation	Roessler 1997a; Hehr 2004
89	c.869G>A	p.Gly290Asp	SHH-C	Nanni 1999; Schell-Apacik 2009; GeneDx new;
90	**c.887G>C**	**p.Gly296Ala**	**SHH-C**	**GeneDx new**
91	**c.928C>T**	**p.Arg310Cys**	**SHH-C**	**Rennes new**
92	**c.961C>A**	**p.Arg321Ser**	**SHH-C**	**NIH new**
93	c.995T>C	p.Val332Ala	SHH-C	Dubourg 2004; Garavelli 2004
94	**c.1015G>T**	**p.Glu339X**	**truncation**	**Rennes new**
95	**c.1037C>T**	**p.Ala346Val**	**SHH-C**	**NIH new**
96	**c.1040C>G**	**p.Pro347Arg**	**SHH-C**	**NIH new, Rennes new**
97	c.1040C>A	p.Pro347Gln	SHH-C	Dubourg 2004
98	**c.1040C>T**	**p.Pro347Leu**	**SHH-C**	**Rennes new**
99	**c.1051C>T**	**p.Gln351X**	**truncation**	**GeneDx new**
100	c.1061T>C	p.Ile354Thr	SHH-C	Dubourg 2004
101	**c.1085C>T**	**p.Ser362Leu**	**SHH-C**	**NIH new**
102	c.1088G>A	p.Cys363Tyr	SHH-C	Richieri-Costa 2006
103	**c.1091A>G**	**p.Tyr364Cys**	**SHH-C**	**Rennes new**
104	c.1117G>A	p.Ala373Thr	SHH-C	NIH new; Hehr 2004
105	**c.1121A>G**	**p.His374Arg**	**SHH-C**	**NIH new**
106	**c.1127C>A**	**p.Ala376Asp**	**SHH-C**	**NIH new**

Subject	cDNA	protein	function	Reference
107	**c.1130T>C**	**p.Phe377Ser**	**SHH-C**	**Regensburg new**
108	c.1132_1140del	p.378_380del	deletion	Nanni 1999
109	c.1142G>C	p.Arg381Pro	SHH-C	Dubourg 2004
110	**c.1145T>C**	**p.Leu382Pro**	**SHH-C**	**Maastricht new**
111	c.1147G>A	p.Ala383Thr	SHH-C	Roessler 1997a; Hehr 2004
112	**c.1171G>A**	**p.Ala391Thr**	**SHH-C**	**Regensburg new**
113	c.1204_1227del	p.402_409del	deletion	Schimmenti 2003
114	c.1213_1227del	p.405_409del	deletion	Nanni 1999
115	**c.1231_1233dup**	**p.Gly411dup**	**duplication**	**NIH new**
116	**c.1246A>G**	**p.Thr416Ala**	**SHH-C**	**NIH new**
117	c.1270C>G	p.Pro424Ala	SHH-C	Nanni 1999
118	**c.1303T>A**	**p.Tyr435Asn**	**SHH-C**	**NIH new**
119	c.1307C>T	p.Ser436Leu	SHH-C	Nanni 1999
120	**c.1366G>C**	**p.Gly456Arg**	**SHH-C**	**NIH new**
121	**c.1370delT**	**p.Met457ArgfsX18**	**frameshift**	**NIH new**

Descriptions of mutations are based on the NM_000193.2 reference sequence, follow HGVS recommendations (www.hgvs.org/mutnomen) and were checked using the Mutalyzer software for the predicted effects on the reference gene (www.lovd.nl/mutalyzer/1.0.1/). Position +1 refers to the A position of the ATG initiation codon for that gene.

Table 2

Chi squared analysis of mutations in different SHH domains.

Domain	Observed	Expected	Statistics, p-value
	Frequency (%)	Frequency (%)	
N terminal			$\chi^2_{(4)} = 214.7$, $p<0.00001$
Identical (*)	28 (70)	100 (50.8)	$\chi^2_1 = 4.2$, $p<0.05$
Similar (:)	4 (10)	34 (17.3)	$\chi^2_1 = 0.8$, $p>0.05$
Related (.)	1 (2.5)	13 (6.6)	$\chi^2_1 = 0.4$, $p>0.05$
Unrelated	7 (17.5)	50 (25.4)	$\chi^2_1 = 0.7$, $p>0.05$
Biologically Equivalent[1]	34 (82.5)	147 (74.6)	$\chi^2_1 = 0.7$, $p>0.05$
Total	40 (100)	197 (100)	
C terminal			$\chi^2_{(4)} = 230.9$, $p<0.00001$
Identical (*)	20 (45.5)	49 (18.5)	$\chi^2_1 = 14.3$, $p<0.001$
Similar (:)	9 (20.5)	29 (10.9)	$\chi^2_1 = 2.3$, $p>0.05$
Related (.)	4 (9.1)	18 (6.8)	$\chi^2_1 = 0.1$, $p>0.05$
Unrelated	11 (25)	169 (63.8)	$\chi^2_1 = 21.8$, $p<0.00001$
Biologically Equivalent[1]	33 (75)	96 (36.2)	$\chi^2_1 = 21.8$, $p<0.00001$
Total	44 (100)	265 (100)	

[1] Includes Identical, Similar, and Related domains as defined by clustalW2 criteria.