The genomic structure, chromosomal localization, and analysis of SIL as a candidate gene for holoprosencephaly

J. D. Karkera, S. Izraeli, E. Roessler, A. Dutra, I. Kirsch, and M. Muenke

Abstract. Holoprosencephaly (HPE) is the most common congenital malformation of the brain and face in humans. In this study we report the analysis of SIL (SCL interrupting locus) as a candidate gene for HPE. Fluorescent in situ hybridization (FISH) analysis using a BAC 246e16 confirmed the assignment of SIL to 1p32. Computational analysis of SIL at the protein level revealed a 73% overall identity between the human and murine proteins. Denaturing high performance liquid chromatography (dHPLC) techniques were used to screen for mutations and these studies identified several common polymorphisms but no disease-associated mutations, suggesting that SIL is not a common factor in HPE pathogenesis in humans.
the lateral plate mesoderm. Either loss of appropriate Nodal expression on the left side of the lateral plate mesoderm, or its bilateral expression, results in a randomization of heart looping (Meyers and Martin 1999; Tsukui et al., 1999). There is presently no clear conserved mechanism described in vertebrates to actually break symmetry, however, factors such as Nodal are thought help to establish left-right asymmetry; furthermore, as a factor correlated with “leftness” its asymmetric expression ensures that the process is nonrandom and associated with characteristic organ positioning (Meno et al., 1998; Meyers and Martin, 1999; Tsukui et al., 1999). In the murine Shh+/− or Smo+/− mutants, Nodal is expressed in the vicinity of the node but is not effectively expressed in the lateral plate mesoderm. This absence of left-sided gene expression is correlated with randomization of heart looping in Shh+/− mutants and is even more severe in Smo+/− mutants where the heart fails to loop in either direction and remains as a straight linear tube. These results suggest an early role of Shh signaling in the control of left-right asymmetry either directly or indirectly through actions impacting on the axial midline (Zhang et al., 2001).

We investigated the human SIL (SCL interrupting locus) gene (Aplan et al., 1991) because the murine Sil+/− mutants displayed features compatible with the HPE spectrum including arrest of neural tube closure and lack of midline separation at the anterior end of the cranial folds leading to holoprosencephaly-like defects. In addition, these mutants displayed left-right developmental abnormalities manifested as abnormal cardiac looping. In Sil−/− mutant embryos the marker Shh displays discontinuous expression accompanied by the lack of Hhf3 expression in the neural tube fold. Heterozygous Sil+/− mice are normal, while homozygous Sil−/− mice die in utero at E10.5. Izraeli et al., also found reduced expression of multiple Shh target genes, despite continued Shh expression in the node and the notochord and suggested a role for Sil in the propagation of Shh signals (Izraeli et al., 1999, 2001). The exact biochemical role of Sil is still unclear due to the absence of homology to any previously known protein. Interestingly, in Ptc+/− and Ptce−/− double murine mutants the expression of multiple Shh target genes, such as Gli2, were markedly reduced suggesting that Sil might be required downstream of Smo in the Shh response pathway (Izraeli et al., 2001).

We hypothesized that a mutation that perturbs the normal function of human SIL might be a risk factor for the development of HPE. Therefore, we decided to study the human SIL gene architecture in detail, to confirm its chromosomal location, and to screen the gene for mutations using denaturing high performance liquid chromatography (dHPLC) in our panel of HPE patients.

Materials and methods

Patients and DNA preparation

DNA samples of patients with HPE were collected by informed consent according to the guidelines of the Institutional Review Board of the NHGRI. DNA was isolated from blood or lymphoblastoid cell lines by standard protocols. A total of 83 probands with familial HPE were chosen for dHPLC screening; this panel is known to be representative of the full spectrum of HPE severity. No patients with laterality phenotypes were available for a similar analysis.

Computational analysis of DNA and protein sequences

Unpublished data (I.K.) pertaining to the genomic organization of SIL was further confirmed by nucleotide homology searches in the public database using the BLASTN program (http://www.ncbi.nlm.nih.gov/blast). The compiled data reflecting the gene structure of all 18 exons were then submitted to GenBank. MultiAlign software (http://www.toulouse.inra.fr/multalin.html) was used for protein homology searches between human and murine SIL. Putative structural motifs were analyzed by PFAM (http://www.sanger.ac.uk/Software/Pfam/search.shtml) and ProfileScan (http://www.isrec.isb-sib.ch/cgi-bin/).

Fluorescent in situ hybridization (FISH) analysis

Slides with normal chromosome metaphase spreads were incubated for 1 h at 37 °C in 2× SSC (0.3 M NaCl and 0.3 M sodium citrate) and then dehydrated sequentially in 70%, 80%, and 90% ethanol. Chromosomal DNA was denatured in 70% formamide, 2× SSC for 2 min at 72 °C followed by dehydration in ethanol washes of 70%, 80%, 90%, and 100%. FISH was performed with the BAC probe 24c616 (Incyte Genomics, MO) labeled with digoxigenin-11-dUTP by nick translation (Boehringer Mannheim, IN) and ethanol precipitated in the presence of 50× Cot-1 human DNA (Lichter et al., 1988). The DNA pellet was resuspended to a final concentration of 25 ng/μl. The hybridization and the post-hybridization step were performed as described earlier (Pack et al., 1999). Slides were counterstained with DAPI, 250 ng/μl (Boehringer Mannheim, IN) with Antifade. Hybridization signals were scored using a Zeiss Axioskop epifluorescence microscope (Zeiss, NY) and the two-color image was captured on a Photometric charge-coupled device camera (Photometrics, AZ) using IP Lab image software (Signal Analytics, VA).

Denaturing high performance liquid chromatography (dHPLC) screening

Amplification of genomic DNA was performed in a 30 μl reaction volume, using 60–100 ng DNA template, 50 μM dNTP, 0.25 μM of each primer, 3 μl of 10× PCR Amplification buffer (Gibco, MD), 1.5 μl 10× Enhancer buffer (Invitrogen, CA), 0.9 μl of 50 mM MgSO4, and 1 U AmpliTaq (Perkin Elmer, CA). All reactions were performed using a PTC-225 thermocycler (MJ Research, MA). Primer pairs and their respective annealing temperatures are described in Table 1. Typical PCR cycling parameters were 95 °C for 4 min followed by 30 cycles at 95 °C for 30 s, annealing at the indicated temperatures for 30 s, extension at 72 °C for 1 min, culminating in a final step of 72 °C for 5 min. One half of the PCR product was used for dHPLC analysis and the other half was retained for direct DNA sequencing.

The dHPLC was performed on a ProStar helix dHPLC system (Varian, CA). In order to enhance heteroduplex formation, the PCR products were denatured at 95 °C for 5 min followed by gradual cooling to 60 °C over 15 min (1 °C/30 sec). The PCR product was automatically injected onto a Helix column and eluted with a linear gradient of buffers A and B (Varian, A: 100 mM triethylamine acetate [pH 7.0], 0.1 mM EDTA, and B: 100 mM triethylamine acetate [pH 7.0], 0.1 mM EDTA, 25%, v/v acetonitrile) at a constant rate of 0.9 ml/min. Each sample was analyzed at the melting temperature (Tm) determined by using the dHPLCMelt software (http://insert.stanford.edu/melt.html). In some instances, two different temperatures were used to enhance the sensitivity of screening. Heterozygous profiles were identified by visual inspection of the chromatograms on the basis of the appearance of additional earlier eluting peaks. Corresponding profiles for the same amplicons, which exhibited only a single peak, could be demonstrated to be homozygous and normal in sequence (data not shown).

Direct DNA sequencing

Amplicons displaying heterozygous profiles were purified using a Qiagen PCR purification kit (Qiagen, CA) and bi-directionally sequenced using the BigDye™ terminator cycle sequencing kit according to the manufacturer’s protocol (Applied Biosystems, CA). Sequencing reactions were analyzed on an ABI 377 automated sequencer.

Nomenclature

Gene mutation nomenclature used in this study follows the recommendations of den Dunnen and Antonarakis (2001). Gene symbols are those following the recommendations of the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk).
Table 1. Primers for SIL mutational analysis

| Exon | Primer | T (°C) (PCR) | Size (bp) | GenBank Accession no. | Tm (°C) (dHPLC) |
|------|--------|-------------|-----------|-----------------------|-----------------|
| 1    | 5’TGTGTTGTGCTACTGACTCCG 3’ | 58 | 163/5’UTR | AF349640 | 64 |
| 2    | 5’TGGATTGTTTATGAGTGGTGGAGGACGACG 3’ | 51 | 5’UTR | AF349641 | 56/61 |
| 3    | 5’GCTGCATATGTGGAACGAGGTCG 3’ | 52 | 154 | AF349642 | 56 |
| 4    | 5’GGAGGTTTTTTTTAGTCAAAGGACCAG 3’ | 51 | 203 | AF349643 | 56 |
| 5    | 5’TTACAGGCGAGACCTCGTC 3’ | 51 | 212 | AF349644 | 57 |
| 6    | 5’GCTGTATTITTTTTATCTCAAGTGAAG 3’ | 55 | 392 | AF349645 | 58 |
| 7    | 5’ACATCTCAGCTTTGTTGTATTTTTGTCG 3’ | 51 | 148 | AF349647 | 54 |
| 8    | 5’CACTGTTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTT
Fig. 3. Examples of selected dHPLC chromatograms of SIL variants. Panels (A), and (C) represent exon 7 containing a 392-bp amplicon analyzed at 58 °C wherein Panel (A) represents the wild type and Panel (C) exhibits T → A in the intron region. Panels (B), and (D) represent exon 13A containing a 632-bp amplicon analyzed at 58 °C wherein Panel (B) depicts wild type and Panel (D) displays A → T in the intron region.

Table 2. Sequence variations in SIL

| Exon | Nucleotide change | Amino acid change | Detections |
|------|------------------|------------------|------------|
| 5    | 257C→T (homozygous) | Ala→Val | Introduces an AccI R.E. site |
| 7    | IVS 7-21T→A (heterozygous) | 5′ intron region | No R.E. site |
| 13   | IVS 13-53A→T (heterozygous) | 5′ intron region | Abolishes an AccI R.E. site |
| 17   | 2874G→A (heterozygous) | Pro→Arg | Introduces an AflIII R.E. site |

* IVS: intervening sequence.
  † R.E.: restriction endonuclease.

Four different heterozygous sequence variations were identified in human SIL (Table 2); however, none were unique to HPE patients. The first homozygous change in exon 5, 257C→T, predicts an A86V mutation and this sequence change introduces an AccI restriction site. The two intron variations detected were present in exon 7 (IVS 7-21T→A) and exon 13 (IVS 13-53A→T). Examples of heteroduplex detection in exons 7 and 13A are shown in Fig. 3. The sequence variation in exon 7 produced a consistent elution profile but in other cases (not shown) distinct sequence variations with a given amplicon produced different characteristic elution profiles. The fourth heterozygous sequence variation, 2874G→A, predicts a substitution P1015R encoded by exon 17 which introduces an AflIII restriction site. Over 200 normal chromosomes were screened either by restriction fragment length polymorphism or by dHPLC for each of the mutations detected in SIL. All the nucleotide variations detected in SIL were also found to be present in the normal control samples.

Discussion

Animal models of brain development have been successfully applied to further our understanding the pathogenetic mechanism of HPE in humans. Sil−/− mutants display severe anterior midline neural-tube defects, abnormalities in left-right axis development, holoprosencephaly-like features, and are embryonic lethal (Izraeli et al., 1999). Furthermore, discontinuous expression of Shh and lack of Hfh3 in the notochord and reduced expression of multiple Shh target genes suggested that Sil deserved to be examined as an HPE candidate gene. In this study, we investigated the sequence variations of SIL in HPE and found no evidence for its role as a common genetic determinant of this disorder in our panel of samples.

Sil−/− murine mutants display a lack of midline separation at the anterior end of the cranial folds resulting in holoprosencephaly-like defects and these embryos ultimately die in utero while the heterozygous mice have no distinct phenotype. Since all of our clinical samples were obtained from live-born infants, it appears likely that our failure to find evidence for a role for SIL in HPE results, in part, from the fact that the phenotype may be autosomal recessive lethal in both mice and humans.
The utilization of a high-throughput approach to mutation screening by dHPLC methodology is extremely sensitive, rapid, and very cost-effective as compared to the traditional single-strand conformation polymorphism (SSCP). Even though our mutation screening did not suggest loss or gain of function of SIL as a common denominator of HPE in humans, nonetheless, we cannot formally exclude the possibility that rare cases could be caused by this mechanism.

The bilateral expression of Nodal, Leftib, and Pitx2, in the lateral plate mesoderm of Sil–/– murine mutants could be attributed to a defective midline barrier, which allows the spread of the left-right signaling cascade across the midline (Izraeli et al., 1999; Bisgrove et al., 2000). Similar observations have been made in zebrafish ntl and flh mutants (Rebagliati et al., 1998) where the generation of the midline barrier and not the generation of left-right signals at the node are affected (Bisgrove et al., 2000). In the zebrafish, cye (nodal) is critical for the regulation of fltl, flt2, and pitx2, and is required for the establishment of cardiac laterality (Yost, 1999). These findings suggest that the genes involved in establishing or maintaining the anterior midline barrier can be associated with the development of brain, heart, and gut asymmetries (Bisgrove et al., 2000).

Targeted mutation of Ebaf (Meno et al., 1998) and Pitx2 (Kitamura et al., 1999) has demonstrated the importance of these genes in left-right axis formation (Casey and Hackett, 2000). Since Ptc–/– murine mutants exhibit left-right development abnormalities associated with a lack of Ebaf expression and bilateral expression of Nodal, Leftib, and Pitx2, we propose that SIL might be an excellent candidate gene to study in laterality patients. Furthermore, the defect appears to be related to Shh signaling since Pich and Gli expression are both reduced in Sil–/– embryos. Therefore, the identification of the gene structure and our strategy to analyze the human SIL gene should facilitate the examination of laterality phenotypes.

Acknowledgements

We thank all of the families for participating in these studies, and the Don and Linda Carter Foundation for their support.

References

Aplan PD, Lombardi DP, Kirsch IR: Structural characterization of SIL, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. Mol Cell Biol 11:5462–5469 (1991).

Bisgrove BW, Esnser JJ, Yost J: Multiple pathways in the midline regulate chordate brain, heart and gut left-right asymmetry. Development 127:3567–3579 (2000).

Casey B, Hacket BP: Left-right axis malformations in man and mouse. Curr Opin Genet Dev 3:257–261 (2000).

Chen Y, Stuhl G: Dual roles for patched in sequestering and transducing hedgehog. Cell 87:553–563 (1996).

Den Dunnen JT, Antonarakis SE: Nomenclature for the description of human sequence variations. Hum Genet 109:121–124 (2001).

Izraeli S, Lowe LA, Bertness VL, Campaner S, Hahn H, Kirsch IR, Kuehn M: Genetic evidence that Sil is required for the sonic hedgehog response pathway. Genesis 31:72–77 (2001).

Izraeli S, Lowe LA, Bertness VL, Goud DJ, Dorward DW, Kirsch IR, Kuehn M: The SIL gene is required for mouse embryonic axial development and left-right specification. Nature 399:691–694 (1999).

Kitamura K, Miura H, Miyagawa-Tomita S, Yanazawa M, Katoh-Fukui Y, Suzuki R, Ohuchi H, Suehiro A, Motoe Y, Nakahara Y, Kondo S, Yokoyama M: Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra-and pericardial mesoderm and right pulmonary isomerism. Development 126:5749–5758 (1999).

Lichter P, Cremer T, Borden J, Manuelidis L, Ward DC: Delineation of human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. Hum Genet 80:224–234 (1988).

Matsunaga E, Shiotto K: Holoprosencephaly in human embryos: epidemiologic studies of 150 cases. Teratology 16:261–272 (1977).

Meno C, Shimoto A, Sajio Y, Yashiro K, Mochida K, Ohishi S, Noji S, Konod H, Hamada H: Lefty-1 is required for left-right determination as a regulator of Lefty-2 and Nodal. Cell 94:287–297 (1998).

Meyers EN, Martin GR: Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. Science 285:403–406 (1999).

Ming HE, Kaupas ME, Roessler E, Brunner HG, Golhi M, Tekin M, Stransky RJ, Suansks E, Bale SJ, Muenke M: Mutations in PATCHED-1, the receptor for SONIC HEDGEHOG are associated with holoprosencephaly. Hum Genet 110:297–301 (2002).

Muenke M, Beachy PA: Holoprosencephaly, in Schaffner DW, Kirsch IR, Kuehn M: The SIL gene is required for the establishment of left-right asymmetry. Dev Biol 199:261–272 (1998).

Roesch E, DeMyer W, Connelly PM, Palmer C, Merritt AD: Holoprosencephaly: birth data, genetic and demographic analyses of 30 families. Birth Defects 11:294–331 (1977).

Roessler E, Muenke M: The structure and function of genes causing human holoprosencephaly. Gene Function Disease 1:1–14 (2000).

Tsukui T, Capdevila J, Tamura K, Ruiz-Lozano P, Rodriguez-Esteban C, Yonei-Tamura S, Magallon J, Chandraratna RA, Chient K, Blumberg B: Multiple left-right asymmetry defects in Shh–/– mutant mice unveil a convergence in the control of lefty-1. Proc natl Acad Sci, USA 96:11376–11381 (1999).

Yost HJ: Diverse initiation in a conserved left-right pathway? Curr Opin Genet Dev 9:422–426 (1999).

Quirk J, van den Heuvel M, Henriques D, Margio V, Jones TA, Tabin C, Ingham PW: The smoothened gene and hedgehog signal transduction in Drosophila and vertebrate development. Cold Spring Harb Symp Quant Biol 62:217–226 (1997).

Rebagliati MR, Toyama R, Haffter P, Dawid IB: Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. Dev Biol 199:261–272 (1998).

Roessler E, Connelly PM, Palmer C, Merritt AD: Holoprosencephaly: birth data, genetic and demographic analyses of 30 families. Birth Defects 11:294–331 (1977).

Tsukui T, Capdevila J, Tamura K, Ruiz-Lozano P, Rodriguez-Esteban C, Yonei-Tamura S, Magallon J, Chandraratna RA, Chient K, Blumberg B: Multiple left-right asymmetry defects in Shh–/– mutant mice unveil a convergence in the control of lefty-1. Proc natl Acad Sci, USA 96:11376–11381 (1999).

Yost HJ: Diverse initiation in a conserved left-right pathway? Curr Opin Genet Dev 9:422–426 (1999).

Zhang XM, Ramalho-Santos M, McMohan AP: Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 105:781–792 (2001).